POST-TRANSLATIONAL MODULATION OF CORTICOSTEROID FEEDBACK INHIBITION IN ADENOHYPOPHYSIAL CORTICOTROPHS

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DECLARATION

This study was carried out primarily under the guidance of Dr Ferenc A. Antoni at the MRC Brain Metabolism Unit, Dept of Neuroscience, University of Edinburgh between October 1995 and November 1998. Dr Michael J. Shipston at the Dept of Biomedical Sciences/Physiology, University of Edinburgh, co-supervised this study. The experimental work presented in this thesis is my own and this thesis has been composed by myself.

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ABSTRACT

Adrenal corticosteroids play a major role in the negative feedback control of the hypothalamic-pituitary-adrenal axis, which ensures that corticosteroid levels are optimal for homeostatic adaptation. The objective of this thesis was to investigate the mechanism of the action of glucocorticoids on stimulus evoked adrenocorticotrophic hormone (ACTH) secretion by anterior pituitary cells *in vitro*. Primary cultures of rat anterior pituitary cells and the mouse corticotroph tumour cell line AtT20 were used.

Early glucocorticoid inhibition was found to require the synthesis of new mRNA and proteins. Previous work indicated that calmodulin is one of the glucocorticoid-induced proteins that may mediate the inhibitory action of glucocorticoid on ACTH secretion. Therefore, AtT20 cells that constitutively over-expressed chicken calmodulin were established to test if elevated calmodulin level could mimic glucocorticoid action. Corticotrophin releasing factor (CRF)-stimulated ACTH secretion by stably transfected AtT20 cells that showed elevated calmodulin mRNA and protein levels and wild type AtT20 cells was found not to be significantly different. Events downstream to the *de novo* synthesis of proteins in glucocorticoid feedback inhibition were studied in cultured rat anterior pituitary cells. Membrane depolarization elicited by a combination of a depolarizing concentration of KCl with 8-(4-chlorophenylthio) adenosine-3',5'-cyclic-monophosphate (CPT-cAMP) or CRF antagonized the

glucocorticoid inhibition of stimulated ACTH secretion. Since the control of membrane potential involves K^+-channels, the effect of K^+-channel blockers on glucocorticoid inhibition was tested. The BK-channel inhibitor, charybdotoxin, had no significant effect on glucocorticoid inhibition, nor did other common K^+-channel blockers (tetraethylammonium, apamin and 4-aminopyridine). Clofilium (IsK-type K^+-channel blocker) and astemizole (an antihistamine with anti-HERG-type K^+-channel properties) were found to significantly reduce the inhibition of the CRF response by glucocorticoid. More specific blockers of IsK-type K^+-channels (chromanol 293B, WAY-123398) or HERG-type K^+-channels (E4031, dofetilide) had no significant effect on glucocorticoid inhibition. Previous studies have also suggested that control of intracellular cAMP levels via Ca^{2+}-dependent feedback is important in glucocorticoid inhibition. Hence, it was tested if clamping of cAMP at high levels could alter glucocorticoid inhibition. High cAMP levels were induced and sustained using arginine vasopressin (AVP) or rolipram (inhibitor of cAMP-dependent cyclic nucleotide phosphodiesterase) in combination with CRF. The glucocorticoid inhibition of ACTH response elicited by CRF/AVP or CRF/rolipram was markedly attenuated. Since protein phosphorylation is integral to cAMP accumulation and cAMP-activated responses, the effect of FK506 (a potent inhibitor of protein phosphatase 2B, calcineurin) was also investigated. In combination with CRF, FK506 elicited an ACTH response that was less effectively inhibited by glucocorticoid.

To summarize, the role of calmodulin as a mediator of glucocorticoid feedback inhibition could not be confirmed in cells AtT20 cells constitutively over-expressing

calmodulin. The data do support the thesis that feedback inhibition by glucocorticoid targets the membrane potential. Furthermore, plasma membrane K^+-channels appear to be involved in glucocorticoid action. However, it was not possible to identify these channels by means of currently available pharmacological agents. The results also show that intracellular cAMP levels modulate the efficiency of glucocorticoid inhibition in normal rat corticotrophs and support the notion that glucocorticoid feedback inhibition may act via amplifying Ca^{2+}-dependent feedback of cAMP.

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AtT20	Mouse clonal corticotroph cell line (subclones: D16:16)
AVP	Arginine vasopressin
bp	Base pairs
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$[Ca^{2+}]_I$	Intracellular free Ca^{2+} concentration
CRF	41-residue corticotrophin releasing hormone
CaM	Calmodulin
cAMP	Cyclic adenosine 3',5'-monophosphate
cDNA	Complimentary deoxyribonucleic acid
cpm	counts per minute
CPT-cAMP	8-(4-chlorophenylthio)3',5'-cyclic adenosine monophosphate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DRB	5,6-Dichloro-1-β-D-ribofluranosylbenzimidazole
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol-bis-(β-amino ethyl ether)N,N'-tetra-acetic-acid
FCS	Foetal calf serum
h	Hour
HPA	Hypothalamic-pituitary-adrenal
Kd	Kilodaltons
K^+	Potassium
min	Minute
mRNA	Messenger ribonucleic acid
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of the mean

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PUBLICATIONS

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INTRODUCTION

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INTRODUCTION

1.1 General considerations

1.1.1 *Stress and the Hypothalamic-Pituitary-Adrenal axis*

A challenge to adaptation from the environment (stressor) activates pleiotropic homeostatic control mechanisms including the immune, central nervous and metabolic control systems, collectively geared towards the restoration of the homeostatic balance of the organism. However, if left unchecked, these powerful stress-induced defence reflexes can overshoot and lead to a collapse of body homeostasis. Consequently, a key function of adrenal corticosteroids (primarily glucocorticoids) released during stress is to terminate these stress-mobilised compensatory defence responses , thereby preventing maladaptation to stress.

The secretion of adrenal glucocorticoids into the peripheral circulation in response to stress is regulated through a classical neuroendocrine negative feedback loop, the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.1). The physiological role of the HPA axis is to tightly regulate plasma glucocorticoid levels in order to maintain cellular homeostasis in response to changes in the environment [see (Dallman *et al.*, 1987; Jones & Gillham, 1988; Keller-Wood & Dallman, 1984) for reviews]. Stress activates the HPA axis, resulting in the release of the major hypothalamic secretagogues, 41-residue corticotrophin releasing factor (CRF) and arginine

Figure 1.1

The Hypothalamic-Pituitary-Adrenal Axis

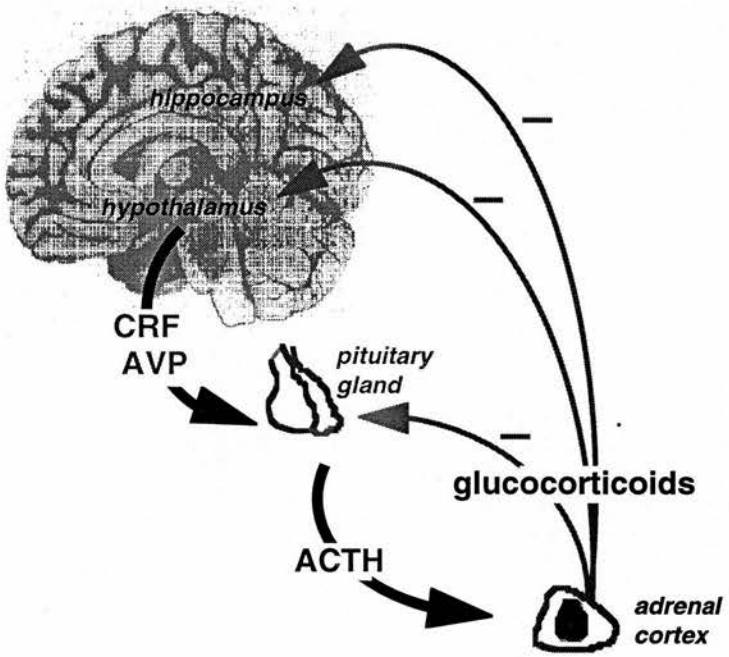

Figure 1.1: 41-residue corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) are the major hypothalamic secretagogues of adrenocorticotrophic hormone (ACTH) from the anterior lobe of the pituitary gland. ACTH in turns stimulates the secretion of glucocorticoids (mainly cortisol in humans and corticosterone in rats) from the cortex of the adrenal gland. Glucocorticoids limit their own production through negative feedback inhibition at the pituitary, the hypothalamus and the hippocampus. – indicates inhibition. See section 1.1.1 for more detailed description.

vasopressin (AVP) into the hypothalamo-hypophysial portal circulation that stimulate the secretion of ACTH from anterior pituitary corticotrophs. (Figure 1.1). Anatomical studies suggest CRF and AVP are produced in the paraventricular nucleus in the hypothalamus via 3 ways (Antoni, 1986; Antoni, 1993; Plotsky, 1988). The parvocellular neurons (which project into the median eminence) are divided into 2 kinds, one expressing CRF exclusively while the other expressing both CRF and AVP. The magnocellular neurons (which project into the posterior pituitary lobe) produce only AVP and may release it into the portal vessels in the median eminence *en passant*. The subsequent ACTH secretion in turn stimulates the release of glucocorticoids (principally cortisol in man, and corticosterone in rats) from the adrenal glands, which affects a wide range of physiological control systems including carbohydrate metabolism, body fluid balance, neural activity as well as anti-inflammatory and immunosuppressive actions (Munck *et al.*, 1984; Munck *et al.*, 1990). Besides counteracting stress-induced homeostatic defence mechanisms, glucocorticoids also limit their own production through feedback inhibition of the HPA axis activity at several major feedback sites, including the anterior pituitary gland, the hypothalamus and further sites in the central nervous system (Sapolsky *et al.*, 1990). Additionally, physiologically relevant hypothalamic inhibitors of ACTH release such as atrial natriuretic peptide may provide further inhibitory control of the HPA axis activity (Antoni *et al.*, 1992; Bierwolf *et al.*, 1998; Fink *et al.*, 1992; Guild & Cramb, 1999).

1.1.2 *Glucocorticoid feedback inhibition*

The regulation of plasma corticosteroid levels is vital since prolonged exposure to glucocorticoids will lead to over-suppression of the stress-activated compensatory responses, which may thwart the maintenance of homeostasis.

Glucocorticoids exert their effects in at least two phases: *early* (occurring within minutes to ~3 h after glucocorticoid exposure) and *late* (requiring >6 h to days) (Shipston, 1992). The *early* phase of glucocorticoid inhibition can be further divided into fast effects (taking place in a matter of seconds to a few minutes) and delayed actions that require several minutes up to 2 h after glucocorticoid administration). Early fast inhibition does not require the interaction of steroid receptors with regulatory sequences in DNA while early delayed action generally involves the induction of new mRNA and protein. Examples of early inhibition include inhibition of ACTH release from anterior pituitary corticotrophs *cf* (Keller-Wood & Dallman, 1984), inhibition of interleukin 1 (IL-1) (Snyder & Unanue, 1982) and IL-2 (Gillis *et al.*, 1979; Gillis *et al.*, 1979) secretion by cells of the immune system (macrophages and T cells respectively) and also suppression of glucose transport in lymphocytes (Munck *et al.*, 1978). Late inhibition generally involves down-regulation of gene-transcription as well as suppression of differentiated cellular functions, for example suppression of proopiomelanocortin (POMC) synthesis in anterior pituitary corticotrophs or induction of apoptosis in lymphocytes (Jones & Gillham, 1988; Keller-Wood & Dallman, 1984; Munck *et al.*, 1984). A recent study showed that in cultures of rat anterior pituitary cells, glucocorticoids can downregulate the mRNA

levels of the CRF type I receptor (in the time frame of late inhibition) (Pozzoli *et al.*, 1996).

In sum, with respect to glucocorticoid feedback inhibition of the anterior pituitary corticotroph, it is well-characterised that late glucocorticoid inhibition results in the depletion of intracellular ACTH stores and downregulation of signalling pathways [see (Dallman *et al.*, 1987; Johnson *et al.*, 1982; Jones & Gillham, 1988; Keller-Wood & Dallman, 1984; Lundblad & Roberts, 1988; McEwen, 1991) for reviews]. In contrast, early glucocorticoid action results in the inhibition of stimulated ACTH secretion through a mechanism that is not well understood other than that it is mediated by newly induced proteins [(Portanova & Sayers, 1974; Shipston & Antoni, 1992; Taylor *et al.*, 1993) and see section 1.3.1].

1.1.3 *Why study the mechanism(s) of glucocorticoid feedback inhibition?*

Glucocorticoids are the key stress hormones responsible for preventing the escalation of the defence mechanisms mobilised during stress. However, excessively high levels of glucocorticoids can have undesirable effects. This may be the reason for the diurnal fluctuations of free cortisol in humans (Baxter & Tyrrell, 1987) and corticosterone in rats (Jacobson *et al.*, 1988) since hormone levels beneficial in the short run may become harmful in the long run. Thus, glucocorticoid-mediated feedback inhibition (which limits its own amounts in circulation) is the physiological process that serves this purpose.

High glucocorticoid levels may over-suppress the production of cytokines for instance (due to the anti-inflammatory and immunosuppressive actions of glucocorticoids (Munck & Guyre, 1991; Munck *et al.*, 1984; Munck *et al.*, 1990)), resulting in the impairment of the immune system. Glucocorticoid excess can also increase the likelihood of diseases like diabetes, hypertension, impotency and myopathy. Furthermore, Cushing's disease and psychiatric disorders like major depression and posttraumatic stress disorder are exemplified by a dysfunctional HPA axis. Cushing's disease is primarily caused by a pituitary tumour (which may or may not hypersecrete ACTH) and is characterised by a disturbance in the corticosteroid feedback mechanism (Burch, 1985). Cushing's syndrome, on the other hand, can be broadly classified as being caused by various kinds of tumours (usually an ectopic ACTH-producing tumour or adrenal cortical tumour) that directly or indirectly results in the hypersecretion of glucocorticoids (Newell-Price *et al.*, 1998). Major depression is characterised biologically by a dysfunctional HPA axis (which can be induced by chronic corticosteroid exposure or stress alone) leading to hypercortisolaemia (Carroll *et al.*, 1981; Sapolsky *et al.*, 1986; Young *et al.*, 1991). Neuroendocrine studies examining the HPA axis under baseline conditions and in response to neuroendocrine challenges have similarly proposed an altered HPA functioning in posttraumatic stress disorder (Yehuda *et al.*, 1991), resulting in cortisol hypersecretion during the period of traumatic stress (Sapolsky, 1997). Thus, better understanding of the mechanisms of glucocorticoid negative feedback inhibition would greatly contribute to any potential development of therapeutic strategies for these diseases.

The murine corticotroph tumour cell line AtT20 is used as a model system for the study of early glucocorticoid feedback inhibition in adenophypophysial corticotrophs. Both cell types are electrically active and their functions are closely coupled to ion channel activity regulated by membrane voltage (Adler *et al.*, 1983; Reisine, 1989; Suprenant, 1982; Kuryshev *et al.*, 1996; Lee & Tse, 1997; Shipston *et al.*, 1996). In this respect, both AtT20 cells and adenohypophysial corticotrophs are systems that could be useful for understanding the actions of adrenal corticosteroids in nerve cells. This is of particular importance since glucocorticoids also exert negative feedback inhibition at the hippocampal level (Meaney *et al.*, 1988; Packan & Sapolsky, 1990; Sapolsky *et al.*, 1990). Furthermore, glucocorticoids (in high concentrations) are known to have deleterious effects on the hippocampus (e.g. causing atrophy of dendritic processes) (Sapolsky, 1996) and may also contribute to HPA dsyfunction in normal aging (Lupien *et al.*, 1998; McEwen, 1999). The paraventricular nucleus of the hypothalamus is also a plausible site of glucocorticoid negative feedback action (Itoi *et al.*, 1987; Kwak *et al.*, 1992; Ogasa *et al.*, 1992; Watanobe & Takebe, 1990). An important function of glucocorticoid feedback at the hypothalamus is to downregulate the production of CRF and AVP (from parvicellular neurons) (Herman *et al.*, 1990; Itoi *et al.*, 1987; Kwak *et al.*, 1992; Ogasa *et al.*, 1992). The decrease in CRF and AVP signals from the hypothalamus subsequently delimit ACTH secretion from anterior pituitary corticotrophs.

In sum, these studies will help to further the understanding of the pathophysiology of psychiatric disorders, in particular, the effects of adrenal corticosteroids on excitable tissues because these are relevant for the actions of steroids in psychiatric disorders.

Furthermore, voltage-operated ion channels feature prominently in the electrophysiological characteristics of adenohypophysial corticotrophs, resembling neurons. As such, study of the role of these voltage-gated ion channels in corticotrophs may contribute to the overall understanding of hormonal action (particularly with respect to the interaction between glucocorticoids and ion channels) in neurons.

1.2 Mechanisms of early glucocorticoid feedback

The early phase of glucocorticoid feedback inhibition involves the induction of newly synthesised proteins. Nonetheless, how these putative glucocorticoid-induced proteins inhibit stimulated ACTH secretion is still not fully understood.

One reason why there is not yet a unifying theme to explain early glucocorticoid inhibition may be due to the differences in the characteristics of the experimental models used and the lack of a clear definition of the time-scale of glucocorticoid exposure employed by different workers. The differences in each study may in turn modulate the magnitude as well as direction of the glucocorticoid (Johnson *et al.*, 1979). For instance, the majority of studies performed to date reports a lag time of 10-30 min after glucocorticoid administration for early inhibition (see section 1.1.2) to be manifest in a variety of *in vitro* corticotroph models (Abou-Samra *et al.*, 1986; Brattin & Portanova, 1977; Dayanithi & Antoni, 1989; Gillies & Lowry, 1978; Mahmoud *et al.*, 1984; Mulder & Smelik, 1977; Portanova & Sayers, 1974;

Widmaier & Dallman, 1984). However, Johnson and colleagues have reported a virtually immediate action of glucocorticoids in AtT20 cells (Johnson *et al.*, 1982) while Familari and Funder failed to find fast or delayed inhibition (sub-components within the time domain of the early phase; see section 1.1.2) in perifused primary cultures of rat corticotrophs attached to cytodex beads (Familari & Funder, 1989). Despite the discrepancies in the literature relating to early glucocorticoid actions, it is of note that in studies investigating inhibition at the pituitary level *in vivo*, glucocorticoids inhibit CRF- and insulin-stimulated ACTH release (within the time domain for early delayed inhibition) in dogs (Keller-Wood, 1990; Keller-Wood & Bell, 1988).

Several lines of evidence suggest that the synthetic glucocorticoid, dexamethasone and the natural corticosteroids, cortisol and corticosterone have different *in vivo* sites of action *cf* (Keller-Wood & Dallman, 1984). Systemic administration of dexamethasone and corticosterone has shown that dexamethasone binding is very high in the pituitary and very low in the hippocampus, while corticosterone binding reflects the reverse situation (De Kloet *et al.*, 1975; McEwen *et al.*, 1976; Rhees *et al.*, 1975). Thus, dexamethasone appears to act predominantly at the pituitary level *in vivo*. However, this differential binding of corticosterone and dexamethasone is much less striking *in vitro* than *in vivo*, suggesting a differential uptake of dexamethasone and corticosterone by brain tissue (De Kloet *et al.*, 1975; McEwen *et al.*, 1976). Recent evidence from adrenalectomized rats and mice suggests that dexamethasone is extruded from brain by the multiple drug resistance (mrd1a) gene-encoded P-glycoproteins (De Kloet, 1997).

Though not yet fully understood, the mechanisms underlying secretagogue-evoked ACTH secretion involve multiple pathways and levels (Shipston, 1992), thus providing numerous sites at which glucocorticoids may exert their inhibitory effect. In particular, the mechanism of early glucocorticoid inhibition is likely to involve the disruption of stimulus-secretion coupling, independent of any effects on ACTH biosynthesis, storage, or degradation (Phillips & Tashjian Jr, 1982). This is evident in the overwhelming majority of studies in which glucocorticoids predominately inhibited stimulated, rather than basal ACTH secretion (Abou-Samra *et al.*, 1986; Clark & Kemppainen, 1994; Shipston *et al.*, 1996; Woods *et al.*, 1992). Studies of early glucocorticoid inhibition in the mouse corticotroph tumour cell line AtT20 (Antoni *et al.*, 1992; Phillips & Tashjian Jr, 1982; Woods *et al.*, 1992) suggests that paracrine interactions between different cell types of the anterior pituitary gland do not appear to play an important role in early inhibition. Consequently, glucocorticoids may directly target DNA elements within the corticotroph to generate the putative proteins that characterise early glucocorticoid inhibition. The following sections discuss the currently proposed mechanisms of early glucocorticoid inhibition in the anterior pituitary corticotroph.

1.2.1 *Modulation of receptor/signal transduction pathways*

Previous work has reported a reduction of CRF plasma membrane binding sites within 10 min of glucocorticoid exposure in primary cultures of enriched rat corticotrophs (Childs & Unabia, 1990). Although such down-regulation is observed in late inhibition (Childs *et al.*, 1986; Pozzoli *et al.*, 1996; Rivier & Vale, 1987;

Schwartz *et al.*, 1986), it is unlikely to be involved in early inhibition. Studies of late glucocorticoid inhibition have reported inhibition of CRF-induced cAMP accumulation (Bilezikjian *et al.*, 1987; Bilezikjian & Vale, 1983) *in vitro*. In contrast, other reports on CRF-stimulated cAMP accumulation *in vitro* and *in vivo* do not support this hypothesis (Giguère *et al.*, 1982; Kant *et al.*, 1989). There is no direct evidence for early glucocorticoid inhibition of CRF or AVP activation of intracellular signal transduction pathways documented to date. Furthermore, ACTH secretion may not necessarily be impaired when CRF receptors and cAMP responses are downregulated *cf* (Aguilera *et al.*, 1986). This suggests considerable plasticity of intracellular signalling mechanisms downstream of G-protein coupled receptor-effector coupling, which could be a site of glucocorticoid feedback inhibition.

Indeed, numerous studies have demonstrated that ACTH secretion stimulated by direct activators of PKA or PKC is inhibited by glucocorticoids *cf* (Oki *et al.*, 1991; Phillips & Tashjian Jr, 1982; Woods *et al.*, 1992). Moreover, glucocorticoids do not prevent cAMP-induced activation of PKA (Miyazaki *et al.*, 1984). Therefore, it is plausible that the site of glucocorticoid inhibition is at a common juncture of stimulus-secretion coupling, downstream to CRF or AVP receptor activation of signal transduction pathways. Since Ca^{2+} plays a pivotal role in the regulation of stimulated ACTH secretion (Antoni, 1986; King & Baertschi, 1990), it is possible that the site of early glucocorticoid inhibition is at or downstream of intracellular free Ca^{2+} mobilisation.

1.2.2 *Suppression of intracellular free Ca^{2+} responses*

The link between a modulatory role for glucocorticoids and calcium handling in corticotrophs was established in early *in vitro* studies [see (Antoni, 1986; Jones & Gillham, 1988; Kraicer *et al.*, 1969)]. In a more recent study (Oki *et al.*, 1991), Oki and co-workers reported, using perifused rat corticotrophs, that the sustained phase of ACTH secretion stimulated by CRF, AVP, oxytocin or combination of secretagogues that is dependent on Ca^{2+} influx through voltage-gated (predominantly dihydropyridine-sensitive, L-type) Ca^{2+}-channels, was inhibited by glucocorticoids. However, the initial phase of secretion elicited by AVP or oxytocin (which was dependent on inositol triphosphate-stimulated intracellular Ca^{2+}) was not sensitive to the inhibitory action of glucocorticoids, implicating the reduction of Ca^{2+} influx through L-type Ca^{2+}-channels by glucocorticoids. In addition, when voltage-dependent Ca^{2+} entry is bypassed in electrically permeabilised AtT20 cells, early glucocorticoid inhibition of Ca^{2+}-mediated ACTH secretion was prevented (Woods *et al.*, 1994). Since early glucocorticoid inhibition can be blocked by depolarization and as voltage-dependent ion channels have a fundamental role in the regulation of ACTH secretion, it is conceivable that changes of intracellular free Ca^{2+} levels ($[Ca^{2+}]_I$) may occur upon treatment with glucocorticoids.

Antoni and co-workers reported evidence for the restriction of Ca^{2+} mobilisation by glucocorticoids in studies examining fura-2 fluorescence measurements of $[Ca^{2+}]_I$ in single AtT20 corticotrophs (Antoni *et al.*, 1992). The synthetic glucocorticoid dexamethasone suppressed CRF-stimulated (but not basal) intracellular Ca^{2+} transients that were dependent on Ca^{2+} influx through L-type Ca^{2+}-channels in

roughly 50% of dexamethasone-treated cells. A similar reduction in CRF-stimulated Ca^{2+} transients was reported in about 25% of single AtT20 cells analyzed after treatment with dexamethasone (Castellino *et al.*, 1992). Another study reportedly found no suppression of intracellular free Ca^{2+} transients in populations of AtT20 cells attached to coverslips (made from the plastic substrate Aclar) after dexamethasone treatment (Clark & Kemppainen, 1994). However, it was not tested whether the inhibitory effect of dexamethasone on ACTH secretion still persisted in Aclar attached cells. Therefore, such a culture system may have altered the characteristics of early glucocorticoid inhibition. Furthermore, it appears that only a proportion of AtT20 cells may secrete ACTH in response to CRF stimulation in single cell studies of ACTH secretion *cf* (Canny *et al.*, 1992). Hence, there may have been a higher proportion of cells not responsive to CRF, resulting in changes of $[Ca^{2+}]_I$ in the functionally relevant cell population being masked.

Overall, the importance of Ca^{2+} transients in the regulation of ACTH secretion and the regulation of Ca^{2+} transients by CRF and glucocorticoids requires further understanding. Nonetheless, it would appear that the studies where the methods for ACTH secretion analysis and intracellular Ca^{2+} recordings can be closely correlated, a change in the characteristics of CRF-induced Ca^{2+} transient after dexamethasone treatment resulting in a reduction of gross $[Ca^{2+}]_I$ over time, is apparent. In addition, reduction of $[Ca^{2+}]_I$ has also been proposed as a mechanism in other models of early glucocorticoid inhibition such as B-lymphocytes (Dennis *et al.*, 1987), basophilic leukemia cells (Her *et al.*, 1990) and pancreatic islet β-cells (Billaudel *et al.*, 1984).

1.2.3 *Hyperpolarization of the membrane potential*

The connection between glucocorticoids and the membrane potential was mooted by studies showing that corticosteroid hormones could modulate hippocampal neuronal excitability. It was demonstrated that glucocorticoids could hyperpolarize the membrane potential within 30 min in hippocampal CA1 neurons (Joels & de Kloet, 1989; Kerr *et al.*, 1989). Such modulation of the plasma membrane potential could indirectly block Ca^{2+} influx, thereby altering the Ca^{2+} homeostasis. A previous electrophysiological study in AtT20 D16:16 cells using whole cell patch clamp recordings has suggested that glucocorticoids limit Ca^{2+} influx indirectly through enhancement of a transient, outward A-type K^+ current (Pennington *et al.*, 1994). Importantly, pharmacological inhibition of K^+ currents with blockers like tetraethylammonium and 4-aminopyridine (which selectively blocks transient A-currents), markedly attenuated the inhibitory action of glucocorticoids on CRF-evoked ACTH secretion and enhances Ca^{2+} influx in AtT20 D16:16 cells (Antoni *et al.*, 1992; Pennington *et al.*, 1994; Wang & Greer, 1995; Woods *et al.*, 1994). The study (Pennington *et al.*, 1994) also demonstrated that ACTH secretion elicited by high extracellular K^+ concentrations was also largely resistant to dexamethasone inhibition in AtT20 cells.

Furthermore, glucocorticoids reportedly enhance other types of K^+ currents within 3h in hippocampal neurons *cf* (Joels & de Kloet, 1992). These include the slow Ca^{2+}-dependent K^+ conductance (I_{AHP}) and the Ca^{2+}-independent K^+ conductances (I_K) (Nicoll *et al.*, 1990). I_{AHP} slowly inactivates the K^+ conductance at the end of a

depolarization period in CA1 pyramidal neurons of the hippocampus while I_K is responsible for the hyperpolarization of CA1 pyramidal neurons (Nicoll *et al.*, 1990). Another study has reported that Kv1 mRNA, encoding a voltage-dependent K^+-channel, was induced by glucocorticoids within 40 min in the anterior pituitary gland as well as clonal GH3 cells. However, no induction was observed in AtT20 (strain not reported) cells (Levitan *et al.*, 1991). Collectively, these data pointed to the possible involvement of K^+-channels in early glucocorticoid inhibition. Recent electrophysiological data from AtT20 cells have shown that selective blockage of BK-type K^+-channels (large-conductance Ca^{2+}-activated K^+-channels) by the synthetic glucocorticoid dexamethasone prevented the early inhibition of ACTH secretion by dexamethasone (Shipston *et al.*, 1996).

1.2.4 *Working Hypothesis : the AtT20 corticotroph model of early glucocorticoid feedback inhibition*

The mouse corticotroph tumour cell line, AtT20 is a potentially useful model system to characterize corticosteroid action in adenohypophysial corticotrophs. Both cell types are electrically active and their functions are closely coupled to ion channel activity regulated by membrane voltage (Adler *et al.*, 1983; Reisine, 1989; Suprenant, 1982). Importantly, AtT20 cells have been shown to retain the main hallmarks of early glucocorticoid inhibition previously observed in rat corticotrophs (Woods *et al.*, 1992). These defining features are onset within 2 h after exposure to glucocorticoids, mediation through Type II glucocorticoid receptors and requirement for mRNA and protein synthesis. However, the main anomaly of the action of glucocorticoids in these tumour cells is that while synthetic glucocorticoids (like

dexamethasone) are effective, natural glucocorticoids (like corticosterone and cortisol) are virtually inactive (Svec, 1984; Svec & Harrison, 1979; Woods *et al.*, 1992). The reasons for this phenomenon are not well understood. Woods and colleagues demonstrated a small but significant inhibition of CRF-stimulated ACTH secretion in AtT20 cells in the presence of glycyrrhetinic acid, an inhibitor of 11β-hydroxysteroid dehydrogenase (11β-HSD) (Woods *et al.*, 1992). 11β-hydroxysteroid dehydrogenase (which exist in two distinct isozyme, 11β-HSD-1 and 11β-HSD-2) catalyses the conversion of the active glucocorticoids corticosterone and cortisol to inert 11 keto-products (11- dehydrocorticosterone, cortisone) (Seckl, 1997; Stewart & Sheppard, 1992). Accumulating evidence suggests that 11β-HSD-1 (the isoform that possesses bidirectional activity) is widely expressed in the brain, including the pituitary (Seckl, 1997). Therefore, it is possible that in AtT20 cells, corticosterone is metabolized into its biologically inactive form by 11β-HSD, accounting for its lack of discernible inhibitory effect on stimulated ACTH secretion. Nonetheless, AtT20 cells on the whole offer a potentially useful starting point for the characterisation of glucocorticoid-induced proteins and their role in the signal transduction pathways underlying early glucocorticoid feedback inhibition *in vivo* (that is in normal corticotrophs).

The following mechanism(s) for glucocorticoid early feedback inhibition using AtT20 cells as a model has been proposed based on experimental evidence described in section 1.2 and elsewhere.

CRF is the major physiological stimulus for ACTH secretion from anterior pituitary corticotrophs (Antoni, 1986). Current evidence suggests that the CRF1 receptor subtype is expressed in normal rat corticotrophs (Chalmers *et al.*, 1995; Pozzoli *et al.*, 1996) as well as in AtT20 cells (Vita *et al.*, 1993). CRF1 receptor is tightly coupled to the Gs-adenylate cyclase cascade and does not bring about any primary changes in polyphosphoinositide metabolism (Xiong *et al.*, 1995). In AtT20 cells, CRF-stimulated cAMP and the subsequent activation of protein kinases by cAMP forms the major intracellular signalling system (Reisine *et al.*, 1985; Schecterson & McKnight, 1993).

Figure 1.2 illustrates the main intracellular signalling pathways regulating CRF-stimulated ACTH secretion and the proposed mechanisms by which early glucocorticoid inhibition occurs in AtT20 cells. Note that although this model is largely derived from work in AtT20 cells, it also incorporates data from normal rat corticotrophs. The major difference between normal and tumoural corticotrophs is the presence of cAMP-activated caffeine/ryanodine-sensitive internal Ca^{2+} stores in normal corticotrophs (Abou-Samra *et al.*, 1987; Won & Orth, 1990). This internal Ca^{2+} pool appears to be negligible in AtT20 cells, which relies exclusively on the influx of extracellular Ca^{2+} to stimulate ACTH secretion (Reisine, 1989; Richardson, 1983; Richardson, 1986).

The sequence of events begins with ligand-bound CRF1 receptor activation of adenylate cyclase mediated through Gs-α. The ensuing cAMP production [demonstrated in normal corticotrophs (Aguilera *et al.*, 1983; Labrie *et al.*, 1982) as

Figure 1.2

Working model of early glucocorticoid feedback inhibition in AtT20 corticotrophs

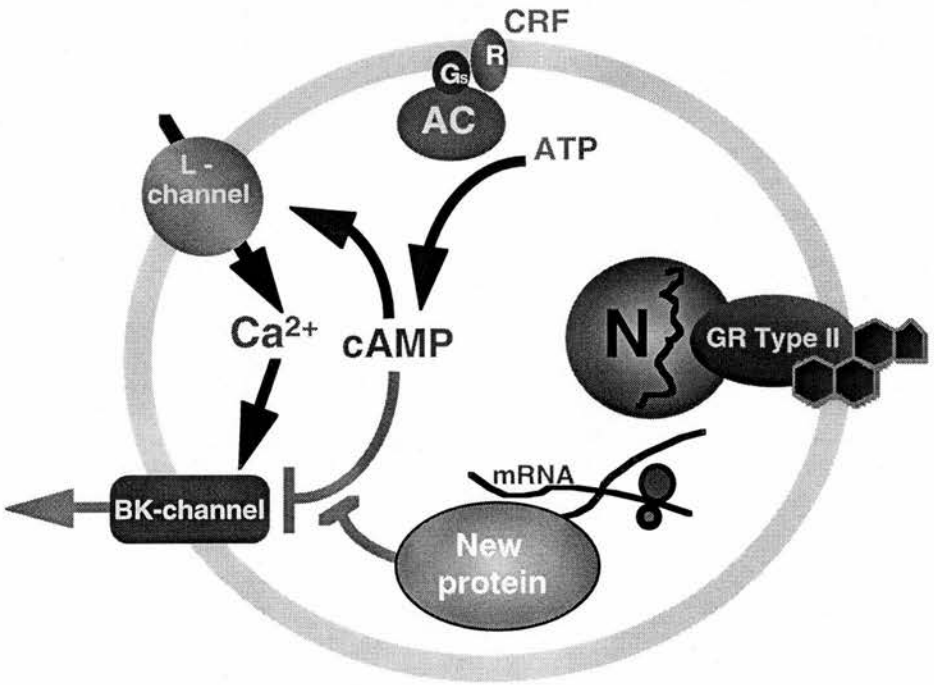

Figure 1.2: *CRF signalling pathway in AtT20 cells:* CRF-receptor (R) coupling induces cAMP production through Gs subunit activation of adenylate cyclase (AC). Subsequent cAMP-dependent phosphorylation activates L-type Ca^{2+} - channel (L-channel) whilst inhibiting BK-channel. The resulting Ca^{2+} influx elicits ACTH secretion. *Glucocorticoid inhibitory pathway:* Glucocorticoid binds to intracellular Type II receptors to induce synthesis of new mRNA(s) and protein(s), which blocks the inhibition of BK-channel caused by cAMP-dependent phosphorylation. ATP: Adenosine triphosphate; N: nucleus; GR: Glucocorticoid receptor. See section 1.2.4 for more detailed description.

well as AtT20 cells (Guild & Reisine, 1987; Litvin *et al.*, 1984; Reisine, 1989)] and accumulation activates the cAMP-dependent protein kinase (protein kinase A, PKA), which has been found to have at least 2 functionally significant targets [many more may well exist (Lory & Nargeot, 1992; Rougon *et al.*, 1989)]. These targets are the L-type Ca^{2+} channels (L-channels) and the BK-type K^+-channels (BK-channels) in AtT20 cells. PKA may activate L-channels increasing the probability of channel opening and prolonging the opening state of L-channels upon depolarization (Wang *et al.*, 1993) through PKA-dependent phosphorylation of either the channel subunits directly or other closely associated channel proteins (Charnet *et al.*, 1995; Puri *et al.*, 1997; Rougon *et al.*, 1989). Modulation of Ca^{2+}-activated K^+-channels by protein phosphorylation (including by PKA) (Ewald *et al.*, 1985; Reinhart *et al.*, 1991) has been extensively documented [reviewed in (Levitan, 1994; Levitan, 1999)]. Specifically, previous work in AtT20 cells suggests that PKA-mediated phosphorylation may inhibit the opening of BK-channels (Shipston *et al.*, 1996). Taking together the effect of PKA on L-channels and BK-channels (Luini *et al.*, 1985; Shipston, 1995; Shipston *et al.*, 1996), it is plausible that the cycling of the membrane potential, which occurs spontaneously at 37°C in AtT20 cells (Adler *et al.*, 1983; Antoni *et al.*, 1992; Suprenant, 1982), is shifted towards depolarization and prolonged Ca^{2+} channel openings (with no significant phosphorylation or G protein-mediated alterations in the function of other ion channels). Note that in normal corticotrophs, there are likely to be additional components of CRF action involving the mobilisation of Ca^{2+} from caffeine-sensitive internal stores (Abou-Samra *et al.*, 1987; Antoni, 1994; Antoni, 1995). The resulting Ca^{2+} influx raises the $[Ca^{2+}]_I$ and triggers the calcium sensor element of the exocytotic apparatus, stimulating the

secretion of ACTH. Although there is evidence that cAMP-mediated phosphorylation acts in synergy with Ca^{2+} to stimulate ACTH release (Guild, 1991), the exact mechanism(s) are not yet fully resolved.

Early glucocorticoid inhibition involves activation of intracellular Type II glucocorticoid receptors (which are ligand-dependent transcription factors) that regulate gene transcription from target genes. Thus, glucocorticoids upon binding to Type II receptors in corticotrophs, induce the synthesis of new mRNAs and proteins, which mediate the inhibitory effects of glucocorticoids. Evidence obtained to date suggests that one such protein is likely to be the multifunctional Ca^{2+}-binding protein, calmodulin. The glucocorticoid-induced protein(s) blocks the inhibition of BK-channels by PKA-mediated phosphorylation (Shipston *et al.*, 1996), thereby allowing the repolarization of the membrane potential mediated by these channels. Consequently, $[Ca^{2+}]_I$ drop as the L-channels close, leading to a cessation of CRF-stimulated ACTH secretion.

A particular aspect of this CRF-activated pathway concerns the intracellular Ca^{2+}-mediated feedback regulation of secretagogue-induced cAMP response. An increase of intracellular cAMP levels elicited by secretagogues (e.g. CRF) produces a rise in $[Ca^{2+}]_I$ known to be essential for triggering ACTH secretion. This rise in $[Ca^{2+}]_I$ in turn downregulates the agonist-induced cAMP response through inhibition of adenylate cyclase and cAMP-degrading phosphodiesterase (Antoni, 1996). Specifically, studies in AtT20 cells has suggested that the targets for this Ca^{2+}-mediated feedback are the type 9 adenylate cyclase (through calcineurin) (Antoni *et*

al., 1995; Paterson *et al.*, 1995) and Ca^{2+}/calmodulin-activated phosphodiesterase (Ang & Antoni, 1996). Additionally, Ca^{2+} may activate voltage-gated K^+-channels (specifically BK-channels) in AtT20 cells (Shipston *et al.*, 1996). This negative mode of Ca^{2+} inhibits cAMP synthesis whilst promoting cAMP degradation, and also cause hyperpolarization of the membrane potential. All these events act in concert to repolarize the membrane potential, downregulate the intracellular cAMP levels (and cAMP-dependent phosphorylation) and Ca^{2+} levels, thereby terminating the secretory response (Antoni, 1996). Given that the early phase of glucocorticoid inhibition is likely to involve calmodulin, it is plausible that early glucocorticoid inhibition involves amplification and sensitization of the cellular machinery to the negative mode of operation of Ca^{2+} (Antoni, 1996).

This section has described putative mechanisms of early glucocorticoid feedback inhibition operable in AtT20 cells, based mostly on work done in AtT20 cells. The precise molecular mechanisms await to be resolved. Furthermore, there may also be other potential sites of action beyond secretagogue-elicited increases in $[Ca^{2+}]_I$ (Shipston *et al.*, 1996).

1.3 Aims and Objectives of the Thesis

The principal aim of this thesis is to investigate in normal rat corticotrophs, the validity of a model of early glucocorticoid feedback inhibition developed in AtT20 cells.

1.3.1 *Role of calmodulin as primary mediator of early glucocorticoid inhibition*

The first hint that the early inhibitory action of glucocorticoids may involve calmodulin came from studies that demonstrated a suppression of intracellular calcium transients by glucocorticoids (Antoni, 1986; Jones & Gillham, 1988). The subsequent search for the mRNAs of calcium-binding proteins in AtT20 cells revealed that steady state mRNA levels for the calmodulin (the multifunctional calcium receptor protein) was significantly increased (Shipston & Antoni, 1992; Woods *et al.*, 1992). The steady state calmodulin mRNAs increased approximately 10-fold within 45 min of dexamethasone treatment while calmodulin protein levels rose 3-fold above control after a 90min exposure to dexamethasone. Current evidence suggests that there are 3 known calmodulin genes in rats (Gannon & McEwen, 1994; Nojima, 1989) as well as in humans (Fischer *et al.*, 1988), which encode the same protein, but are the products of different genes with distinct 5' and 3' non-coding region. Interestingly, the glucocorticoid-inducible calmodulin appears to be largely specific to Type II calmodulin mRNA , which induction can be blocked by protein synthesis inhibitors (Shipston, 1992). Recent reports suggest that the approximately 1.6kb mRNA transcript detected in AtT20 cells (Shipston, 1992; Shipston & Antoni, 1992) is likely to correspond to the rat Type I calmodulin gene (Sola *et al.*, 1996; Ye & Berchtold, 1997), and not the rat Type II calmodulin gene.

The calmodulin mRNA transcripts corresponding to the other 2 rat genes (Type II and III) were only present in very low levels which precluded accurate quantification (Shipston, 1992).

Other previously proposed potential mediators of early glucocorticoid inhibition, namely calbindin, chromogranin A and lipocortin (annexin) I were not found to be induced by dexamethasone within the time frame of early glucocorticoid inhibition by protein immunoblots and Northern blot hybridization studies for known sequences of these proteins (Antoni *et al.*, 1992; Woods *et al.*, 1992). The actin cross-linking protein, caldesmon, has also been associated with glucocorticoid inhibition since it was observed that glucocorticoids increased the cellular content of this protein in AtT20 cells. However, this induction was apparent only after more that 6 h of glucocorticoid exposure, beyond the time interval for early glucocorticoid inhibition (Castellino *et al.*, 1992).

Induction of calmodulin by glucocorticoids has been proposed as a generic mechanism involved in early glucocorticoid inhibition of calcium signalling in several systems. These include lymphocytes (Baughman *et al.*, 1991; Dowd *et al.*, 1991) and corticotrophs (Antoni *et al.*, 1992; Shipston & Antoni, 1992). Although direct evidence for a primary mediatory role for calmodulin is still not available, several indirect lines of evidence suggest that calmodulin may indeed have a central role in early glucocorticoid inhibition. Firstly, glucocorticoids induce elevation in calmodulin mRNA and protein levels in AtT20 cells within the time frame of early glucocorticoid inhibition. Conversely, the simultaneous exposure of AtT20 cells to

glucocorticoids and CRF prevented glucocorticoid inhibition of stimulated ACTH secretion as well as glucocorticoid induction of calmodulin mRNA (Shipston & Antoni, 1992). Secondly, the inhibition of calcineurin (calmodulin-activated phosphatase), which has been proposed as an important feedback inhibitor of calcium action in excitable cells (Armstrong, 1989), antagonises early glucocorticoid inhibition in AtT20 cells ((Shipston *et al.*, 1994) and see section 4.3.1) Finally, a recent study (Ribar *et al.*, 1995) has demonstrated a direct role for elevated calmodulin levels in the inhibition of stimulated insulin secretion from pancreatic β-cells.

This indirect evidence clearly suggest a role for calmodulin in early glucocorticoid feedback inhibition in corticotrophs. Therefore, this first part of the thesis attempts to provide confirmation for the role of calmodulin in the inhibitory action of glucocorticoids through over-expressing calmodulin in AtT20 cells. This will be done in 2 ways, by generating constitutive calmodulin over-expression as well as inducible calmodulin over-expression systems in AtT20 cells. These over-expression systems will then be used to study both basal and stimulated ACTH secretion to see if artificially elevated calmodulin levels have any effect on ACTH secretion in AtT20 cells.

1.3.2 *Role of potassium channels in glucocorticoid inhibition*

Previous work in AtT20 cells has shown that dexamethasone treatment enhances a fast activating and transient outward (A-type) K^+ current by a mechanism requiring protein synthesis. The enhancement of this voltage-regulated K^+ current may blunt

the effects of depolarization on the voltage-gated L-channels (Pennington *et al.*, 1994). Importantly, pharmacological studies in AtT20 cells has demonstrated that inhibition of K^+ currents (including A-type K^+ currents) attenuates the inhibitory action on CRF-evoked ACTH secretion and enhances Ca^{2+} influx (Antoni *et al.*, 1992; Pennington *et al.*, 1994; Wang & Greer, 1995; Woods *et al.*, 1994). These studies indicated that the apparent glucocorticoid suppression of increases in $[Ca^{2+}]_I$ stimulated by CRF could be mediated through K^+-channels, in particular, Ca^{2+}-activated K^+-channels. This class of K^+-channels has been suggested to be closely associated with Ca^{2+}-channels in the plasma membrane [in a variety of voltage-operated secretory systems; (Gola & Crest, 1993)], and may therefore be involved in the localised repolarization (hyperpolarization) of membrane potential following membrane depolarization and Ca^{2+} influx (Heyward *et al.*, 1993; Iijima *et al.*, 1990; Robitaille & Charlton, 1992). Analysis of the K^+ and Ca^{2+} currents in dexamethasone-treated AtT20 cells (Pennington *et al.*, 1994; Shipston *et al.*, 1996) have indicated that BK-channels are modulated by dexamethasone. Shipston *et al.* demonstrated that CRF and CPT-cAMP inhibited an outward current by reducing the open probability of BK-channels (Shipston *et al.*, 1996). This effect was apparently mediated by PKA since CPT-cAMP is an activator of PKA and that the pretreatment of AtT20 cells with Rp-cAMPS (a PKA inhibitor) significantly diminished this effect of CRF. Importantly, dexamethasone pretreatment abolished the inhibitory effect of both CRF and CPT-cAMP on the K^+ current. Furthermore, dexamethasone inhibition of ACTH release was completely reversed by relatively potent BK-channel inhibitors such as iberiotoxin (Shipston *et al.*, 1996) and charybdotoxin (Woods *et*

al., 1992). These data therefore strongly suggest that BK-channels are common targets of cAMP and glucocorticoid-induced proteins in AtT20 cells.

In normal rat corticotrophs, CRF-activated signalling pathways involve the cAMP-dependent PKA pathways (Kuryshev *et al.*, 1995; Kuryshev *et al.*, 1995; Lee & Tse, 1997). Several lines of evidence suggest that K^+-channels are also likely to be important in the action of early glucocorticoid feedback inhibition of stimulated ACTH release in normal corticotrophs. For instance, preliminary studies in rat anterior pituitary cultures showed that elevation of extracellular KCl concentrations reduced the efficiency of dexamethasone inhibition (Antoni, 1996). Other depolarizing agents (such as the sodium channel blocker, veratridine) could also elicit ACTH secretion that was partially (Antoni & Woods, 1992) or completely resistant to dexamethasone inhibition (Halilimanabat *et al.*, 1995). Furthermore, glucocorticoids have also been found to modulate voltage-gated K^+-channel mRNA expression in the anterior pituitary (Levitan *et al.*, 1991) and also in heart (Levitan *et al.*, 1996; Takimoto & Levitan, 1994).

Consequently, the aim of this part of the thesis is to examine the role of K^+-channels in early glucocorticoid inhibition in non-tumoural corticotroph cells. In particular, whether BK-channels are also essential components of the early glucocorticoid inhibitory pathway. Experiments will be chiefly carried out in primary cultures of rat anterior pituitary cells. The effects of K^+- channel blockers on secretagogue-(mainly CRF) induced ACTH secretion will be investigated.

1.3.3 *Role of protein phosphorylation in glucocorticoid inhibition*

One of the plausible sites for glucocorticoid inhibition is early in the CRF-activated signalling pathway. The generation of cAMP signals (see section 1.2.4) by CRF serves to propagate the activation signals through cAMP-dependent phosphorylation by PKA. Thus, blockade of this cAMP-activated phosphorylation step will truncate the signal transduction pathway induced by CRF, resulting in a disruption of the ACTH secretory process. Protein phosphatases are plausible candidates that could reverse the effects of protein phosphorylation. The main types of protein phosphatases (PP), PP1, PP2A, PP2B (calcineurin) and PP2C have all been previously described in AtT20 cells (Antoni *et al.*, 1993). Of note is the Ca^{2+}/calmodulin-activated protein phosphatase 2B, known as calcineurin. It has been shown to dephosphorylate a specific region of the RII subunit of PKA, which is a poor substrate for the other protein phosphatases (Blumenthal *et al.*, 1986). Therefore, it is conceivable that calcineurin may even modulate PKA activity. In wider terms, calcineurin may modulate PKA-phosphorylated targets like the L-type-Ca^{2+} channels, the BK-channels (section 1.2.4) or some other as yet undefined component that acts in synergy with Ca^{2+} to elicit ACTH secretion (Guild, 1991).

The immunosuppressants FK506 and cyclosporin A (CsA) are potent inhibitors of calcineurin (Liu *et al.*, 1991). FK506 and CsA must bind to their intracellular immunophilins, FK-binding protein-12 (FKBP12) and cyclophilin A respectively prior to binding and inhibiting calcineurin activity (Liu *et al.*, 1991).

Previous studies have indicated that calcineurin has an inhibitory role in stimulus-secretion coupling in AtT20 cells since FK506 and CsA stimulated ACTH secretion by AtT20 cells (Antoni *et al.*, 1993). Subsequent studies have shown that FK506 enhanced the ACTH secretory response to CRF and that this secretion was less effectively inhibited by the synthetic glucocorticoid (Shipston *et al.*, 1994). The effect of FK506 on dexamethasone-inhibited ACTH release was blocked by the FK506 antagonist drug, L685,818 (Shipston & Antoni, unpublished). These data provided strong pharmacological evidence that calcineurin played an important role in early glucocorticoid inhibition of stimulated ACTH secretion in AtT20 cells (Antoni *et al.*, 1995; Antoni *et al.*, 1993).

As part of a concerted effort to elucidate the molecular mechanisms of early glucocorticoid inhibition in normal rat corticotrophs, the role of calcineurin in ACTH secretion and glucocorticoid inhibition of stimulated ACTH release was investigated in primary cultures of rat anterior pituitary cells. Northern and Western blot analysis has revealed that FKBP-12 and cyclophilin A mRNA and protein is present in rat anterior pituitary tissue (Ohye *et al.*, 1998). Therefore, the use of FK506 and CsA as pharmacological probes will shed light on any potential role that calcineurin may have in glucocorticoid inhibition in normal rat corticotrophs.

1.3.4 *Summary*

In sum, the principal aim of this thesis is to examine and verify the validity of a model of the cellular mechanism(s) underlying early glucocorticoid established in mouse corticotroph tumour cell, AtT20. The specific aims are as follows:

1) To confirm the hypothesis that early glucocorticoid feedback inhibition involve the induction of calmodulin., which acts as the primary mediator of the inhibitory actions of glucocorticoids.

2) To investigate, in normal (non-tumoural) rat corticotrophs,, the role of K^+-channels in the signal transduction pathway of early glucocorticoid inhibition. In particular if BK-channels, which have been found to be an integral part of glucocorticoid inhibition in the AtT20 system, are also important components of glucocorticoid inhibition in normal corticotrophs.

3) To examine the role of protein phosphorylation, specifically the involvement of calcineurin (calcium-activated protein phosphatase) in glucocorticoid inhibition in normal corticotrophs.

2

MATERIALS AND METHODS

2

MATERIALS AND METHODS

2.1 *Materials*

2.1.1 *Animals*

Male Wistar rats (120-200g) obtained from Charles River Ltd (Margate, Kent, UK) were used for all the experiments with cultured rat anterior pituitary cells. The rats were caged singly (to minimise stress-induced activation of the hypothalamic-pituitary-adrenal axis).with free access to pelleted food and tap water in a light (lights on at 0500h , off at 1900h) and temperature-controlled (22°C) environment 24 h prior to the setting up of primary cultures. On the day of preparation of primary cultures (see section 2.2), rats were routinely sacrificed by decapitation before 1030h.

2.1.2 *AtT20 cells, mouse anterior pituitary corticotroph tumour cell line*

AtT20 D16:16 cells used were originally obtained from Dr. S. L. Sabol, NIH, Bethesda, Maryland, USA (Sabol, 1980). Cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Life Technologies Ltd, Paisley, UK) containing 4.5g glucose/L and supplemented with 10% foetal calf serum (FCS, Harlan Sera-lab, Sussex, UK) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were passaged every 7 days after reaching 70-80%

confluency in 75cm^2 flasks (Costar, High Wycombe, Buckshire, UK) using Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS, GibcoBRL, Life Technologies Ltd, Paisley, UK) containing 0.1% w/v EDTA. After pelleting at 200g for 5 min, 1 x 10^6 cells were reseeded into a 75cm^2 flask. All experiments were performed on cells between passage 16 and 30.

2.1.3 *Biochemicals*

Steroids: Corticosterone (RU 3039) was from Roussel-Uclaf (Romainville, France), dexamethasone was from Sigma-Aldrich (Poole, Dorset, UK). Working steroid stocks were dissolved in dimethylsulphoxide (DMSO) at a concentration of 10mM and stored at -20°C before dilution in the appropriate cell incubation medium on the day of experiment.

Peptides: Rat 41-residue corticotrophin-releasing factor (CRF), arginine vasopressin (AVP) and human adrenocorticotrophin hormone ($ACTH_{1-39}$) were obtained from Bachem UK Ltd (Saffron Walden, Essex, UK) or Peninsula Laboratories Ltd (St Helens, Merseyside, UK). Working stock peptides were stored as 0.1mM (CRF), 1mM (AVP) or 0.25µM ($ACTH_{1-39}$) stocks in 0.01M HCl (including 1mM ascorbate where appropriate to prevent methionine oxidation) at -70°C. Bovine brain calmodulin was from Sigma and was kept as 20µM stock in 0.1% w/v bovine serum albumin (BSA, Sigma A-4378) at -40°C.

K^+-channel inhibitors: Tetraethylammonium hydrochloride (TEA) and 4-aminopyridine (4-AP) were obtained from Aldrich Chemical Co. (Gillingham,

Dorset, UK). Scorpion charybdotoxin was from Peptide Institute Inc. (Scientitic Marketing Associates, Barnet Herts., UK), apamin (from bee venom) and astemizole were from Sigma while clofilium tosylate was from RBI (Sigma, UK). E4031 was a generous gift from Eisai Chemical Co. Ltd (Kashima-gun, Ibaraki-ken, Japan) while dofetilide (UK-68,798) was generously provided by Pfizer Central Research, Sandwich, UK (Gwilt *et al.*, 1991). WAY-123,398 was a generous gift from Dr Walter Spinelli, Wyeth-Ayerst Research, Princeton, NJ, USA (Spinelli *et al.*, 1992) while chromanol 293B was generously provided by Dr H.-J. Lang, Hoechst A.G., Frankfurt/Main, Germany (Busch *et al.*, 1996).

Stock solutions of the K^+-channel inhibitors stored at -20°C were as follows: 500mM TEA, 10mM E4031, 0.1mM charybdotoxin (stored at -70°C) and 1mM apamin were made up in UV-irradiated double distilled water; 10mM dofetilide in acidified saline; 100mM 4-AP and 10mM WAY-123,398 in 0.1M HCl; astemizole, clofilium and chromanol 293B in DMSO.

Primary antisera: Sheep anti-ACTH antiserum was a generous gift from Prof PJ Lowry, University of Reading, Berkshire, UK. Rabbit anti-cAMP antiserum (AL-43) was generously supplied by Dr A Baukal and Dr KJ Catt (NICHD, National Institutes of Health, Bethesda, MD, USA).

cDNA clones: A cDNA clone for normal and mutant chicken calmodulin (0.76kbp insert in NcoI/XbaI site of pCaMPL (Putkey *et al.*, 1987)) was generously provided by Prof AR Means (Dept of Pharmacology, Duke University Medical Centre, North

Carolina, USA). A cDNA clone for chicken calmodulin (0.28kbp insert in HindIII site of pGEM-1) was a generous gift from Dr DR Dowd, Arizona Cancer Center, Tuscon, Arizona, USA (Dowd *et al.*, 1991).

Molecular biology reagents: TRIZOL® Reagent (GibcoBRL) was used for single-step isolation of total RNA. Bacto-tryptone, bacto-yeast extract and agar were obtained from Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK. All restriction enzymes and nucleotides were from Boehringer Mannheim UK (Lewes, East Sussex, UK), Pharmacia Biotech (St. Albans, Hertfordshire, UK) or GibcoBRL. All other reagents were from Sigma (Molecular Biology Grade) unless otherwise stated.

Miscellaneous: (-)BayK8644 (RBI), puromycin, D-ribofluranosylbenzimidazole (DRB), doxycycline and activator-deficient bovine brain cyclic-nucleotide phosphodiesterase 1 (PDE1) were from Sigma. Rolipram was from Schering A.G. (Berlin, FRG). The following compounds were generous gifts from the corresponding companies: FK506 (Fujisawa GmbH, Munich, Germany); cyclosporin A (Novartis, UK); L685,818 (Merck, Rahway, NJ, USA). 8-(4-chlorophenylthio)adenosine-3',5'-cyclic-monophosphate (CPT-cAMP) was from Biolog Life Science Institute (Bremen, FRG).

Stock solutions of the following compounds and the corresponding storage temperature: 5mM (-)BayK8644 (-20°C) and 100mM rolipram in DMSO (-40°C); 2mM FK506, 2mM cyclosporin A and 50mM L685-818 in ethanol and stored at -

70°C; 33mM DRB in ethanol (4°C); 50mM CPT-cAMP and 37mM puromycin were dissolved in UV-irradiated double distilled water and kept at -40°C.

All other materials were obtained as described in the relevant methods or results section. General chemicals were from BDH (Poole, Dorset, UK) or Sigma and of the highest analytical grade available.

2.2 *Primary culture of rat anterior pituitary cells*

After decapitation of the rats (see section 2.1.1), the anterior pituitary glands were collected under sterile conditions and placed in a plastic Petri dish so that the ventral surfaces of the glands contacted the bottom of the dish. The glands were chopped twice (the second time after 90° rotation of the specimen platform) on a hand-operated McIlwain tissue-chopper setting 0.5mm and lowest blade force. The tissue segments were incubated in HBSS without Ca^{2+} and Mg^{2+} (GibcoBRL) supplemented with 25mM HEPES pH7.4, 0.15mM $CaCl_2$, 0.15mM $MgSO_4$, 0.5mg/ml trypsin, 0.01μg/ml DNase I (Sigma) and 0.25% w/v BSA (Sigma A-7906) for 30min at 37°C under constant shaking at 250 cycles/min on a IKA Vibrax orbital shaker. Following trypsinisation, 200μl of 10 kallikrein unit/ml Trasylol® (Bayer, UK) was added and the tissue segments were triturated with a 1ml Gilson pipette tip attached to the end of a 5ml polystyrene pipette for 10 min. The suspension was then filtered through a 70μM nylon mesh and centrifuged at 200g for 10 min. The pelleted cells were resuspended in 5ml HBSS supplemented with 25mM HEPES

pH7.4, 0.15mM $CaCl_2$, 0.15mM $MgSO_4$, 0.01μg/ml DNAse I, 0.25mg/ml soybean trypsin inhibitor (Sigma), recentrifuged and suspended in DMEM (containing 4.5g glucose/L) supplemented with 2.5% FCS (Harlan Sera-lab) and 7.5% horse serum (Sigma H-1138). Cell viability as assessed by Trypan Blue exclusion was over 95%. Approximately 5 x 10^4 cells per well were plated in 24 -well plates (Falcon) and cultured in DMEM containing 2.5% FCS and 7.5% horse serum or serum-free media (DMEM containing insulin-transferrin-sodium selenite media supplement (Sigma), 42μg/ml fibronectin (Sigma) and 3% BSA). Cells were cultured in a humidified incubator with 95% air and 5% CO_2 at 37°C for 4 days before being used for ACTH secretion experiments.

2.3 *Static ACTH secretion experiments*

Primary cultures of rat anterior pituitary cells or AtT20 cells were cultured in 24 -well plates (see section 2.2) for 4 and 5 days respectively before being used for ACTH secretion studies.

Figure 2.1 is the schematic representation of a typical experimental procedure for an ACTH secretion experiment. Prior to experimental manipulation, cells were washed twice and maintained in DMEM containing 25mM HEPES (pH7.4) and 0.25% BSA for 1 h at 37°C. Cells were pretreated with steroids or vehicle for 2 h at 37°C after which secretagogues were applied. Generally, drugs like K^+-channel blockers, L-

Figure 2.1

Outline of typical experimental procedure for ACTH secretion experiments

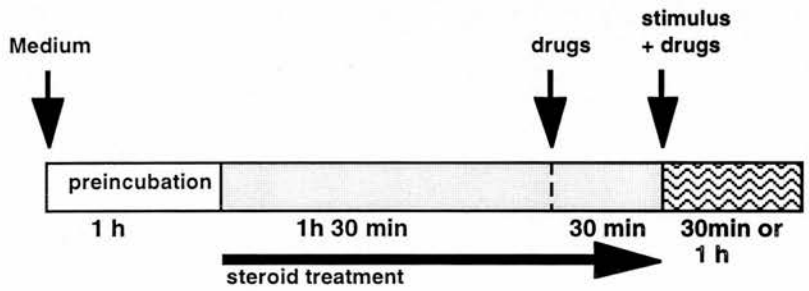

Figure 2.1: Schematic representation of a generic procedure for ACTH secretion experiments. Cells were preincubated in DMEM (Medium) at 37°C for 1 h after being washed twice in DMEM. Steroids (either dexamethasone ot corticosterone) was administered for 2 h at 37°C. In experiments where drugs (e.g. K^+-channel blockers and Ca^{2+}-channel activators) were used, cells were treated with the respective drugs during the last 30 min of steroid pretreatment, before cells were challenged with stimulus (CRF, AVP or CPT-cAMP). The corresponding drugs were again administered during stimulation of ACTH secretion by the secretagogue (stimulus) for 30 min or 1 h, depending on the cell type used (see section 2.3).

channel activators, Ca^{2+}-channel inhibitors and immunosuppressants were introduced 30 min before and during stimulation with secretagogues (refer to respective Figure legends for the treatment protocol of other drugs). The duration of secretagogue challenge was 30 min and 1 h for experiments with AtT20 cells and cultured rat anterior pituitary cells respectively. Cells were challenged with secretagogues at 37°C in a shaking water bath to enhance ACTH release. Medium containing the secreted ACTH was collected and spun briefly at 200g to pellet any floating cells and the supernatant used for determination of ACTH by radioimmunoassay.

2.3.1 *Measurement of ACTH secretion*

Duplicate 50µl aliquots of experimental medium was assayed for immunoreactive ACTH using a double antibody precipitation radioimmunoassay (Dayanithi & Antoni, 1989) after appropriate dilution in RIA buffer [0.05M sodium phosphate buffer, pH7.4, 0.1% BSA, 0.1% Triton-X-100, 2.5mM EDTA and 100 kallikrein inhibitor units (KIU) of aprotinin per ml].

Fifty microlitres of sheep ACTH antiserum (courtesy of Prof P.J. Lowry, University of Reading) at a final titre of 1:100,000 in RIA buffer containing 6% polyethylene glycol 6000 (PEG-6000), were mixed with 50µl of sample in a polypropylene microtiter RIA vial (Sarstedt, Sevelen, Switzerland). The assay mix was incubated with 10µl of ^{125}I-ACTH (typically between 12,000 to 15,000 cpm in 10µl sodium phosphate buffer without PEG-6000) for 18 h at 4°C. Subsequently, donkey anti-sheep IgG (Scottish Antibody Production Unit, SAPU, Lanarkshire) and non-

immune rabbit serum (SAPU) were added to a final titre of 1:25 and 1:400 respectively and incubated for 3 h at 4°C. Four hundred microlitres of ice-cold 6% PEG-6000 were added and the bound label separated from free by centrifugation at 1,950g for 25 min at 4°C. The resultant supernatant was decanted, tubes washed and blot-dried, and the radioactivity remaining in the pellet counted on a Packard autogamma counter.

Standard curves were constructed in the range 0.125 to 32.0 fmol ACTH/50µl using human $ACTH_{1-39}$ diluted in DMEM or RIA buffer as appropriate. Non-specific binding was determined by either incubating in the presence of excess unlabelled ACTH or by omitting the ACTH antiserum and was typically <10% of the total bound counts. Inter- and intra-assay coefficients of variance were typically <10% and <5% respectively.

ACTH content was determined by interpolation from the % Bound counts *vs* log ACTH concentration curve (see Figure 2.2), or its logit transformation, generated using the respective algorithm of the Packard Cobra autogamma analysis package. Specific bound counts were typically 35% of total counts applied. Percent bound counts (%B) is defined as:

$$\frac{X-NSB}{Bo-NSB} x100\%$$

Figure 2.2

ACTH concentration standard curve

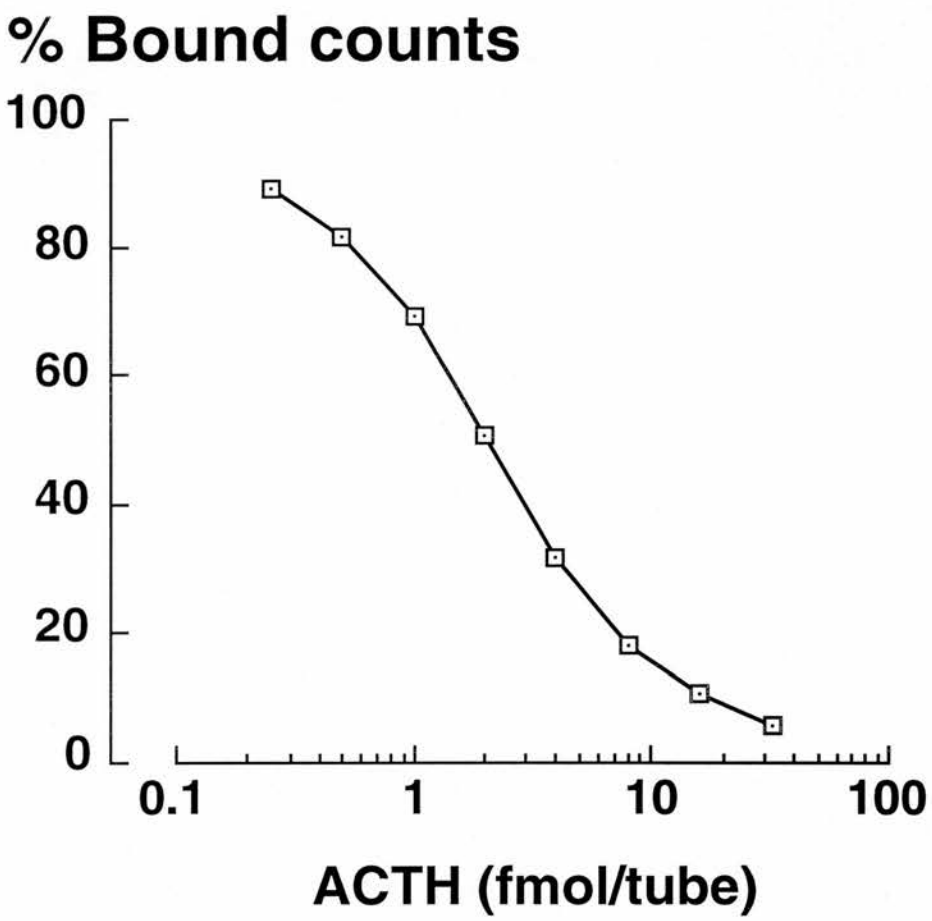

Figure 2.2: ACTH concentration standard curve using sheep anti-ACTH at a final dilution of 1:100,000, generated using the RIA method described in section 2.3.1.

Where X= cpm for standard/unknown sample, B_o = cpm in the absence of ACTH and NSB is the non-specific cpm.

Logit %B is defined as:

$$\log \frac{\%B}{100-\%B}$$

2.4 *cAMP accumulation experiments*

Cells used for cAMP accumulation experiments were prepared similarly as described for ACTH secretion experiments (section 2.2 and 2.3). Prior to experimental manipulation, cells were washed twice and maintained in DMEM (see section 2.3) for 1 h at 37°C after which drugs were introduced for 30 min before fresh drugs and secretagogues were applied for various lengths of time at 37°C. cAMP production was terminated by addition of HCl to a final concentration of 0.1M. Total cellular cAMP was extracted by one cycle of freeze-thawing in 0.1M HCl and determined by radioimmunoassay.

2.4.1 *Measurement of cAMP accumulation*

Duplicate 50µl samples of cell extract in 0.1M HCl were assayed for immunoreactive cAMP using a double antibody precipitation reaction (Dufau *et al.*, 1973) using cAMP antiserum, Cab4 (courtesy of Dr KJ Catt, NIH, Bethesda, USA) at a final dilution of 1:100,000. All assay reactions were performed in polypropylene

tubes (Alpha-Labs) in 50mM sodium acetate buffer, pH6.0 containing 0.25% BSA and 10% sodium azide. Samples were incubated overnight at 4°C with AL43, typically between 12,000 and 15,000 cpm of ^{125}I-cAMP, donkey anti-rabbit IgG (1:30 final dilution, SAPU) and non-immune rabbit serum (1:200 final dilution, SAPU). Subsequently, 700µl of ice-cold 6% PEG-6000 was added and the bound label separated from free by centrifugation at 1,950g for 25 min at 4°C. Non-specific binding was typically <10% of total bound counts, approximately 40% of total counts bound to the antiserum. Inter- and intra-assay coefficients of variance were typically <10% and <5% respectively. Standard curves were generated in the the range 0.78 to 400 fmol cAMP/50µl using cAMP (Sigma) as standard. Assayed cAMP content was as determined as described for ACTH radioimmunoassay (section 2.3.1).

2.5 *General molecular biology methods*

All routine molecular biology techniques such as restriction digestion, gel electrophoresis, cloning etc. were performed as described in (Sambrook *et al.*, 1989).

2.5.1 *Total RNA extraction*

Total RNA was extracted using TRIZOL® Reagent (GibcoBRL), a mono-phasic solution of phenol and guanidine isothiocyanate, based on the single-step guanidinium thiocyanate-phenol-chloroform RNA isolation method. Approximately 10^7 AtT20 cells (70-80% confluency) were lysed directly as a monolayer from a $75cm^2$ flask using 5ml of the TRIZOL® Reagent and homogenised by trituration in a sterile polypropylene tube (Falcon). After 5 min incubation at room temperature to permit the complete dissociation of nucleoprotein complexes, 1 ml of chloroform was added and shaken vigorously for 15 seconds before incubation at room temperature for a further 3 min. Samples were centrifugated at 12,000g for 15 min at 4°C and the aqueous phase from the resultant supernatant was transferred to a fresh tube to which 2.5ml of the isopropanol was added and incubated at room temperature for 10 min. After centrifugation at 12,000g for 10min at 4°C, the RNA pellet was washed in 75% ethanol (made up in sterile diethylpyrocarbonate (DEPC)-treated water) and recentrifuged at 12,000g for 5min at 4°C. The resulting RNA pellet was dried and resuspended in 50µl of DEPC-treated water. RNA quality and quantity was determined using the absorbance ratio at 260 and 280nm and by ethidium bromide staining of agarose gels.

2.5.2 *Random prime labelling of cDNA inserts*

cDNA inserts were cut overnight from vector DNA using the appropriate restriction enzymes (Sambrook et al., 1989) and gel purified on a 0.7% low-melting point agarose (GibcoBRL) gel run in 1X TAE (40mM Tris, 20 mM acetic acid, 1mM EDTA, pH 8.0) buffer. The excised insert was heated at 100°C in 100µl of UHP (Ultra High Purity) water until the gel just melted and subsequently used for labelling reaction using the Ready-to-Go® DNA labelling kit (Pharmacia). For the labelling reaction, sixty-five nanograms (determined by spectrophotometry at OD_{260nm}) of heat denatured DNA insert was used as template in a reaction volume of 50µl containing the reconstituted Reaction Mix from the Ready-to-Go® kit and 50µCi α^{32}P-dCTP (DuPont). The reaction was carried out at 37°C for 30 min and terminated by the addition of 50µl of stop solution (40mM EDTA). Labelled DNA was purified by Sephadex G-25 column chromatography (Nu-Clean, IBI Ltd, Cambridge, UK) according to the manufacturer's instruction and used directly in hybridization. DNA was typically labelled to a specific activity of $>1x10^9$ cpm/µg.

2.5.3 *Northern blotting and hybridization*

Total RNA was denatured for 15 min at 65°C in MOPS/EDTA buffer (20mM MOPS, pH 7.0 (3-(N-morpholino)propanesulfonic acid) containing 0.66M formaldehyde, 5mM sodium acetate and 1mM EDTA). Samples were electrophoresed for 3 h at 90V on a 1% agarose gel (GibcoBRL) containing 0.66M formaldehyde using MOPS/EDTA as running buffer (Founey et al,1987). After equilibrating the gel in 10X SSC (1XSSC : 150mM NaCl, 15mM trisodium citrate, pH7.0) for 5 min, RNA was transferred overnight at 4°C by capillary blotting to

nitrocellulose (Schleicher & Schull, Dassel, West Germany) using 10X SSC. Subsequently, RNA was fixed onto nitrocellulose membrane by baking for 2 h at 80°C before use in hybridization.

Northern blots were typically prehybridized for 2 h at 55°C in 50% v/v deionized formamide (Sigma), 5X SSC, 2X Denhardts (Sigma), 0.1% w/v SDS and 0.2mg/ml salmon sperm carrier DNA (Sigma). Labelled DNA was applied directly to the prehybridization mixture (typically $2x10^6$ cpm/ml) and incubated at 50°C for 16-18h in a hybridization chamber. Filters were washed twice with 1X SSC, 0.1% SDS at 50°C for 15min and then with 0.1X SSC, 0.1% SDS for a further 15 min. Subsequently, filters were exposed at -70°C to Kodak Hypermax beta film using intensifying screens for 3 days before autoradiographs were developed.

2.5.4 *Transformation of competent E.Coli cells*

Competent bacteria cells were generated using the $CaCl_2$ method (Sambrook *et al.*, 1989). Fifty millilitres of pre-equilibrated LB-broth were inoculated with 0.5ml of an overnight 50ml culture of bacteria (JM-109) and incubated at 37°C with shaking to an OD_{600} of 0.2. Flasks were cooled on ice and bacteria pelleted at 2,500g for 5 min at 4°C. The pellet was resuspended in 25ml of ice cold 50mM $CaCl_2$ and incubated at 4°C for 4 h and subsequently used for transformation with plasmid DNA. Typically, 20ng of plasmid DNA was incubated for 30 min on ice with 200µl of competent bacterial cells, followed by heat shock treatment for 2 min at 42°C, cooled on ice and plated overnight at 37°C on LB-agar plates containing 50µg/ml ampicillin.

2.5.5 *Plasmid DNA minipreps*

Minipreps of plasmid DNA were prepared from 3 ml overnight bacterial cultures in LB-broth (10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl per litre) containing 50μg/ml ampicillin using the Akaline Lysis method (Sambrook *et al.*, 1989). Cultures were pelleted and resuspended in 100μl of ice-cold solution 1 (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA), followed by addition of 200μl of solution 2 (0.2M NaOH, 1% SDS) to lyse the cells and 150μl of solution 3 (3M potassium, 5M acetate). Cell debris was pelleted at 12,000g at RT for 5 min and nucleic acids were separated from proteins by applying 450μl of phenol/chloroform and centrifugation (12,000g, RT, 2 min). DNA was precipitated from the resultant supernatant (aqueous phase) with an equal volume of isopropanol followed by centrifugation at 12,000g for 5 min at RT. The DNA pellet was washed with 75% ethanol, dried and resuspended in 50μl of sterile UHP water containing 20μg/ml RNase A and stored at -40˚C.

2.5.6 *Generation of uni-directional cDNA inserts*

The uneven termini of double stranded cDNA were blunted using 9U of Klenow in a 40μl reaction mix containing 0.5M Tris-HCl, pH7.6, 0.1M MgCl2 and 25μM each of dATP, dCTP, dGTP and dTTP at 37˚C for 15 min. The blunted cDNA inserts were extracted using the Geneclean® II kit and restriction digested with Xba I. This generates cDNA with a 5' blunt end and a 3' Xba I recognition site that allows uni-directional insertion of the cDNA into the pcDNA3 vector.

2.5.7 *Ligation of cDNA into plasmid vectors*

Typically, 0.5μg of cDNA was ligated to 10μg of calf intestinal alkaline phosphatase-treated pcDNA3 or pUHC10-3 vectors to achieve optimum ligation. Ligation was carried out in a 20μl reaction containing: 10mM Tris-acetate, 10mM magnesium acetate, 50mM potassium acetate, 1mM ATP and 9 Weiss units of T4 DNA ligase, for 16 to 18 h at room temperature. Ligation reactions (10μl) were used to transform competent JM-109 (see section 2.5.4).

2.6 *Generation of constitutive and inducible calmodulin over-expression systems in AtT20 cells*

A schematic outline of the methodology for generating stable AtT20 cell lines that constitutively overexpress calmodulin (CaM) or contain inducible calmodulin over-expressing constructs in shown in Figure 2.3.

For the constitutive over-expression system, wild type or mutant [CaM-8, 8 amino acids deleted and therefore has lost its ability to activate its downstream targets, but can still bind Ca^{2+}; (Ribar *et al.*, 1995)] chicken CaM cDNA fragment was subcloned into pcDNA3 (a mammalian expression vector from Invitrogen). Recombinant CaM-containing pcDNA3 constructs were transfected into AtT20 cells by electroporation. Transfected AtT20 cells were selected with 0.6mg/ml geneticin

(G418, GibcoBRL) for typically about 10 to 12 weeks before stablly transfected AtT20 cell lines were obtained.

For the inducible expression system, the same wild type or mutant CaM cDNA fragments used in the constitutive over-expression system, were subcloned into the pUHC10-3 vector, part of the reverse tetracycline (rTet) inducible expression system (Gossen *et al.*, 1995). AtT20 cells were first transfected with the vector containing the rTet transcription activator moiety pUHD17-3) and selected with 0.6mg/ml G418. Subsequently, the CaM-containing puHC10-3 vector (obtained by ligation, see section 2.5.7) was co-transfected with a puromycin-resistant vector into AtT20 cells already stably transfected with pUHD17-3. These AtT20 cells were selected with both puromycin and G418 to ensure that both the pUHC10-3 (which contains the CaM cDNA fragment) and pUHD17-3 vectors (which contains the rTet moiety) are present in these stably transfected AtT20 cells.

Figure 2.3

Generation of constitutive and inducible over-expression systems in AtT20 cells

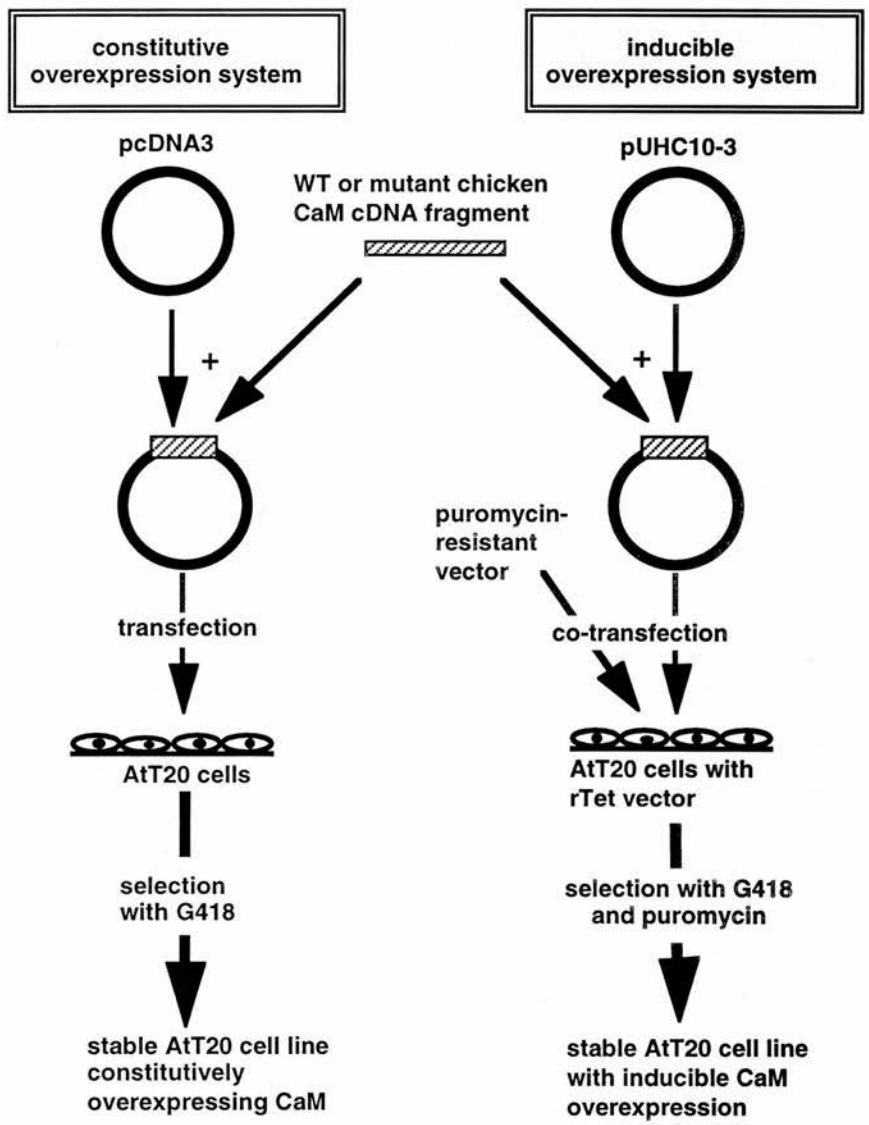

Figure 2.3: See section 2.6 for full description of the methodology of creating these stable AtT20 cell lines.

2.7 *Miscellaneous*

2.7.1 *Measurement of calmodulin*

2.7.1a Preparation of cell extracts

Stably transfected AtT20 cells were stripped and pelleted in HBSS at 200g for 5 min at 4°C. Cell pellets were resuspended in homogenisation buffer containing 20mM Tris-HCl, pH7.5, 1mM EGTA, 200 KIU/ml aprotinin and 2mM phenylmethylsulfonyl fluoride (1.5-2 X 10^7 cells/ml) and lysed by one cycle of freeze-thawing in liquid nitrogen and 30°C water bath. The homogenate was centrifuged at 2,000g for 10 min, followed at 30,000g for 20 min at 4°C. Any intrinsic cyclic nucleotide phosphodiesterase (PDE) activity was eliminated by heat inactivating the supernatant at 95°C for 5 min, followed by centrifugation at 30,000g for 20 min at 4°C to yield the cytosolic fraction. The amount of protein was quantitated using Coomassie Protein Assay Reagent (Pierce, Chester, UK) with BSA as standard based on the method of (Bradford, 1976).

2.7.1b Calmodulin assay

Calmodulin content was measured by minor modifications of a radiometric assay (Schilling, 1994) for quantitating PDE activity based on the precipitation of cAMP hydrolysis product, 5'-AMP, with $ZnSO_4$-$Ba(OH)_2$ treatment (Krishna, 1968). The PDE assay buffer contained 100mM Tris-HCl, pH7.5, 5mM MgSO4, 0.2mM Ca^{2+}, 10µl of cell extract (approximately 10µg protein, Section 2.6.1a) and 0.2U/ml of

bovine brain PDE (Sigma), in a total volume of 150µl. After preincubation at 30°C for 10 min, the reaction was initiated by the addition of 50µl substrate (containing a final concentration of 100µM cAMP and 0.2µCi/ml [2,8-^{3}H]cAMP) and incubated at 30°C for 15 min. The reaction was terminated by the addition of 100µl of 21.5mM $ZnSO_4$ followed by 100µl of 17.5mM $Ba(OH)_2$ to precipitate the 5'-AMP formed. The suspension was subsequently filtered over Whatman GF/C filters on a vacuum manifold, and washed twice with 3ml of 1mM NaOH/100mM NaCl. Radioactivity retained on the filters was measured by liquid scintillation counting with 10ml of Emulsifier-SAFE™ scintillation cocktail (Packard Bioscience, Pangbourne, Berkshire, UK). Assays for background levels of calmodulin were carried out using the homogenisation buffer (see section 2.7.1a) in place of cell extracts.

2.7.2 *Analysis of results*

Each experiment was carried out at least twice with **n** replicates for each treatment within each experiment. All data are presented as means±SEM and were analyzed by 2- or 1-way analysis of variance followed by Dunnett's test, Newman Keuls' test or contrast of means where appropriate. Results presented as percentage of control ACTH release were derived from the raw data as follows: (X-B)/(C-B) X 100%, where B refers to the basal ACTH secretion, C refers to ACTH secretion elicited by the control stimulus and X refers to ACTH secretion elicited by the control stimulus in the presence of dexamethasone or corticosterone.

3

ANALYSIS OF THE PUTATIVE ROLE OF CALMODULIN IN MEDIATING EARLY GLUCOCORTICOID FEEDBACK INHIBITION OF STIMULATED ACTH RELEASE IN AtT20 CELLS

3

ANALYSIS OF THE PUTATIVE ROLE OF CALMODULIN IN MEDIATING EARLY GLUCOCORTICOID FEEDBACK INHIBITION OF STIMULATED ACTH RELEASE IN AtT20 CELLS

3.1 *Introduction*

Early (<2h) glucocorticoid inhibition of stimulated ACTH release in anterior pituitary corticotrophs involves the rapid induction of new mRNA and proteins (Arimura *et al.*, 1969; Brattin & Portanova, 1977; Dayanithi & Antoni, 1989; Woods *et al.*, 1992) that suppress intracellular free Ca^{2+} transients (Antoni *et al.*, 1992). Previous studies in AtT20 cells have demonstrated that the synthetic glucocorticoid dexamethasone significantly raised the levels of steady state calmodulin mRNA by 90 min after application of the steroid to AtT20 cells (Shipston & Antoni, 1992). This suggested that calmodulin may be one of the proteins newly induced by glucocorticoids. Therefore, this section of the thesis serves to verify the putative role of calmodulin as a mediator of early glucocorticoid feedback inhibition in AtT20 cells and attempts to define the mechanisms of calmodulin action. For instance, is calmodulin acting as a Ca^{2+} buffer to disrupt Ca^{2+} homeostasis or is the effect of calmodulin exerted through Ca^{2+}/calmodulin activated enzymes. To address this, AtT20 cells constitutively over-expressing calmodulin or a mutant form of

calmodulin (CaM-8) that only displays Ca^{2+} binding (see section 2.6 for the methodology of creating these novel AtT20 cell lines) were generated. The cell lines that stably over-expressed calmodulin were then analyzed for their ability to mimic the inhibitory effect of glucocorticoid on stimulated ACTH secretion by studying the characteristics of basal and CRF-stimulated ACTH secretion in these novel calmodulin over-expressing AtT20 cells.

Results

3.2 Characterisation of calmodulin over-expression in calmodulin-transfected AtT20 cells

3.2.1 *Northern analysis of calmodulin mRNA in stably transfected AtT20 cells*

AtT20 cells stably transfected with recombinant mammalian expression vector pcDNA3 containing calmodulin or CaM-8 were characterised initially by analysis of mRNA expression. Figure 3.1 shows the different amounts of a ~1.6kb mRNA species that hybridized to a chicken calmodulin probe. Of the calmodulin-transfected AtT20 cell lines, CaM2 (Figure 3.1) showed the strongest hybridization signal at ~1.6kb, indicating the constitutive expression of elevated calmodulin mRNA. The hybridization signal was weak in wild type AtT20 cells (Figure 3.1) because endogenous calmodulin mRNA is low. Moreover, the chicken calmodulin probe does not cross react with endogenous mouse calmodulin mRNA under the high stringency conditions used for the hybridization (see section 2.5.3).

3.2.2 *Measurement of functional calmodulin levels in transfected AtT20 cells*

The amount of functional calmodulin protein was assessed by measuring the activity of a Ca^{2+}/calmodulin activated cyclic nucleotide phosphodiesterase (PDE) (described in section 2.7.1b). The transfected AtT20 cell line (CaM2, see Figure 3.1) in which apparently elevated levels of calmodulin mRNA were detected, the functional calmodulin levels were significantly higher than in wild type AtT20 cells, control pcDNA3-transfected and mutant CaM-8 transfected AtT20 cell lines (Figure 3.2).

The calmodulin levels measured in CaM2 were 302±32% of that measured in wild type AtT20 cells (designated 100%)(mean of 3 experiments, n=3, $p<0.05$ when compared to wild type AtT20 cells, Student's 2-tailed, unpaired t-test).

The analysis of calmodulin mRNA and protein levels in CaM2 has indicated that this transfected AtT20 cell line is constitutively over-expressing functional calmodulin and was thus analyzed further in functional ACTH secretion studies.

Figure 3.1

Characterisation of calmodulin mRNA expression in wild type and stably transfected AtT20 cells

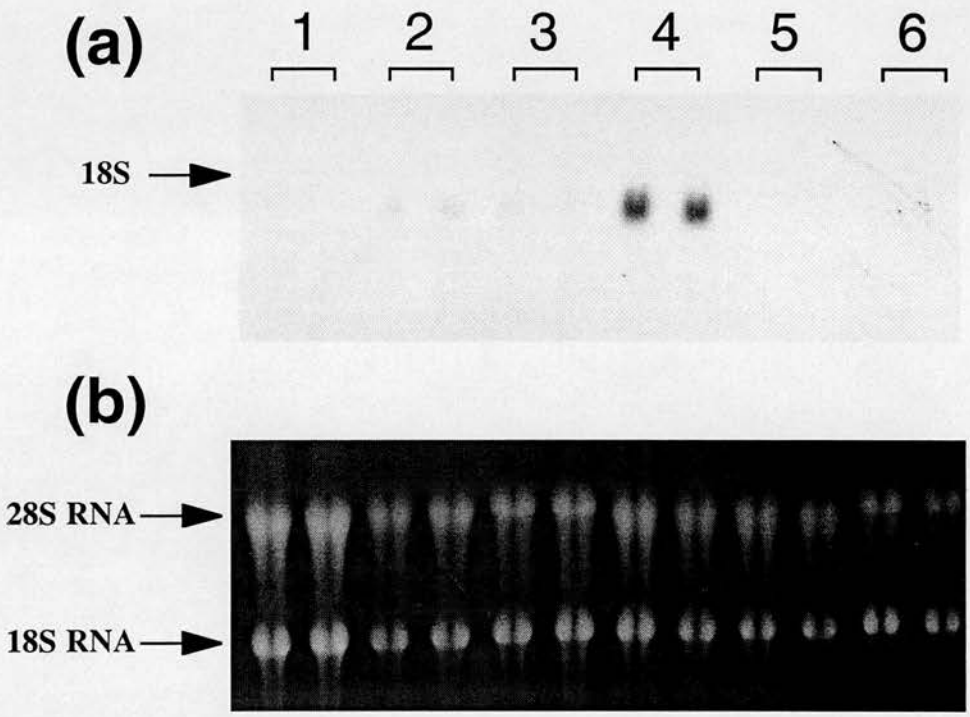

Figure 3.1: (a) Northern blot for calmodulin mRNA in duplicate lanes for: (1) AtT20 cells transfected with recombinant mammalian expression vector pcDNA3 containing mutant calmodulin (CaM-8) cDNA; (2), (3) and (4) different clones of AtT20 cells transfected with recombinant pcDNA3 containing chicken calmodulin cDNA; (5) AtT20 cells transfected with pcDNA3 alone and (6) wild type AtT20 cells. **NB**: (4) is designated CaM2 for further reference. Total RNA for each cell line was run in duplicate lanes and hybridised with a (α^{32}P) dCTP labelled 0.28kbp HindIII fragment of chicken calmodulin cDNA (pGMCaMc). Blots were hybridised as described in section 2.5.3, autoradiographs were exposed for 3 days. (b) Ethidium bromide-stained gel electrophoresis of total RNA (20μg/lane) of the corresponding cell lines in (a) on 1% agarose gel for 3 h at 90V (as described in section 2.5.3).

Figure 3.2

Characterisation of functional calmodulin protein levels in wild type and transfected AtT20 cells

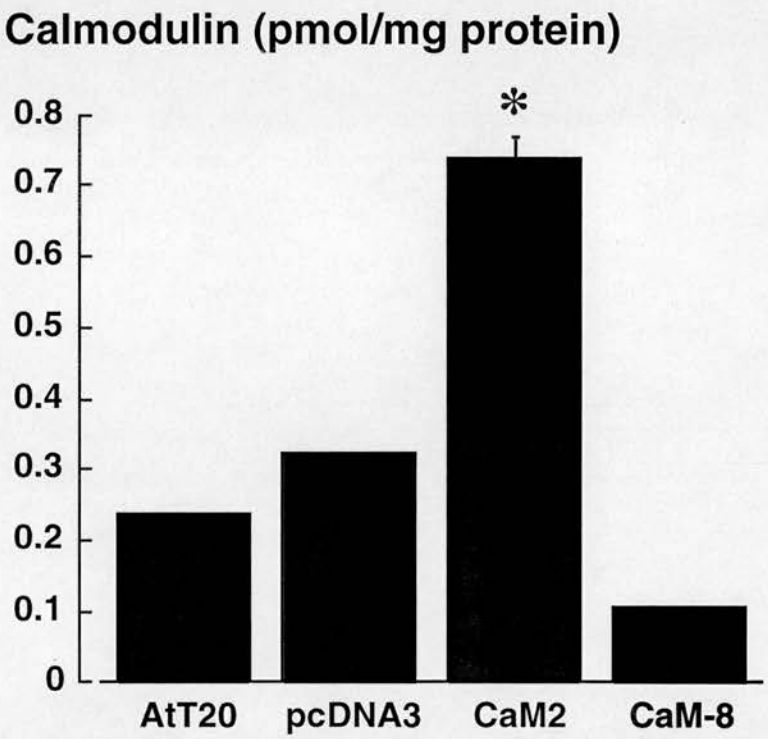

Figure 3.2: Amounts of calmodulin measured in wild type AtT20 cells (AtT20), AtT20 cells transfected with a mammalian expression vector pcDNA3 (pcDNA3), with recombinant pcDNA3 containing calmodulin cDNA (CaM2) or containing a mutant calmodulin cDNA (CaM-8). Solid columns are means, bars indicate SEM, n=3/group. The functional calmodulin levels were measured with a Ca^{2+}/calmodulin-activated PDE assay (described in section 2.7.1b) and standardised to cytosolic protein content (defined in section 2.7.1a). *P<0.01 when compared to calmodulin level measured in AtT20, 1-way ANOVA followed by Newman-Keuls test. Where error bars are not visible, the SEM bars are too small to be seen on this scale. Results shown are representative of 3 measurements.

3.3 Characteristion of CRF-stimulated ACTH release in stably transfected AtT20 cells

In order to investigate whether the constitutive over-expression of calmodulin in AtT20 cells could mimic the early onset inhibition of stimulated ACTH release by glucocorticoid, ACTH release by CaM2 in static incubation studies were carried out.

3.3.1 *Response to CRF*

The variation of the absolute amounts of ACTH release by wild type and transfected AtT20 cells precluded direct statistical comparison (see figure legend of Figure 3.3 for the absolute basal values in fmol/well). Therefore, 10nM CRF-stimulated ACTH release from wild type and transfected cells was expressed as percentages of basal ACTH release within each cell type. The ACTH response to 10nM CRF was not significantly different among wild type AtT20, control pcDNA3-transfected AtT20 and CaM2 cells (Figure 3.3).

In summary, the data above show that the apparent constitutive expression of elevated calmodulin levels in the calmodulin over-expressing AtT20 cell line CaM2, was not correlated with any significant modulation of CRF-stimulated ACTH release.

Figure 3.3

Evaluation of CRF-stimulated ACTH release in wild type and transfected AtT20 cells

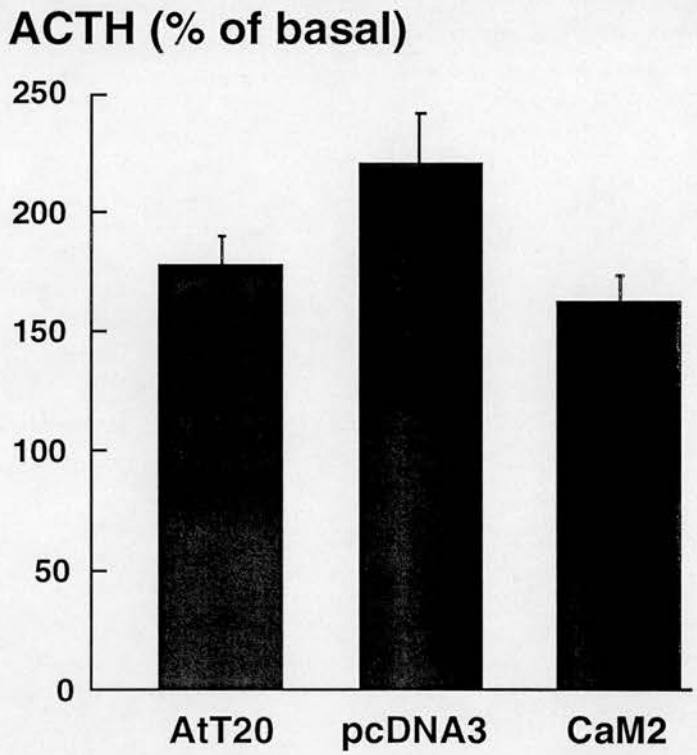

Figure 3.3: ACTH release elicited by 10nM CRF in wild type AtT20 cells (AtT20), AtT20 cells transfected with pcDNA3 and with recombinant pcDNA3 containing calmodulin cDNA (CaM2). Solid columns are means, bars indicate SEM, n=3-4/group and expressed as % of 10nM CRF-induced ACTH release over basal release within each of the respective cell lines. Basal release in fmol/well (means±SEM) from AtT20, pcDNA3 and CaM2 cell lines respectively ---- ***Expt 1:*** 417.0±13.8, 424.8±35.1, 573.6±5.8 ; ***Expt 2:*** 764.3±69.0, 628.7±35.8, 1045.1±41.5 ; ***Expt 3:*** 143.8±15.5, 211.1±14.8, 134.8±9.44. Results shown are mean data from 3 experiments.

3.4 Preliminary study of an inducible expression system in AtT20 cells

Results from section 3.3.1 indicated that constitutive over-expression of calmodulin appears to have no discernible effect on CRF-induced ACTH release in AtT20 cells. Instead, an inducible expression system may be more appropriate for mimicking the induction of new proteins by glucocorticoids during the early onset of feedback inhibition. To address this issue, the reverse tetracycline repressor (rTet) -regulated inducible expression system developed by Gossen and colleagues (Gossen *et al.*, 1995) was tested for its suitability for use in AtT20 cells. As the tetracycline derivative doxycycline was a more potent agent for inducing gene expression in this Tet system, its effects on ACTH secretion were examined in normal AtT20 cells.

3.4.1 *Doxycycline reduced CRF-stimulated ACTH release*

An optimal concentration of doxycycline (1μg/ml), based on previous studies by Gossen and colleagues (Gossen & Bujard, 1992), was used to treat ATt20 cells for 30 min or 5 h. CRF (10nM) was applied to stimulate ACTH secretion after the various lengths of doxycycline treatment. Doxycycline (1μg/ml) had no effect on basal ACTH release but significantly reduced the ACTH response to 10nM CRF (Figure 3.4).

These results suggested that the use of the Tet inducible expression system in AtT20 cells would not be appropriate for studying the characteristics of ACTH secretion.

Figure 3.4

Doxycycline reduces CRF-elicited ACTH release in AtT20 cells

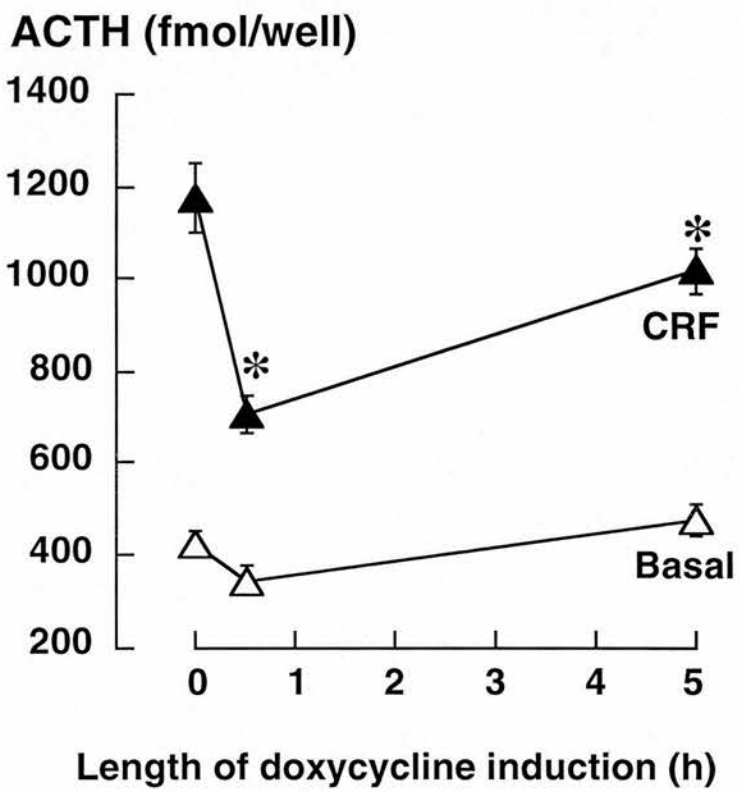

Figure 3.4: Effect of 1μg/ml doxycycline on the ACTH secretory response elicited by 10nM CRF. AtT20 cells were treated with doxycycline for the specified length of time before the cells were challenged with 10nM CRF or vehicle. Cells that received no doxycycline treatment were incubated with vehicle solution for 5 h before stimulation with 10nM CRF or vehicle. Data represent means±SEM, n=4/group. *P<0.05 when compared to control (i.e. no doxycycline treatment). Where error bars are not visible, the SEM bar is less than the space required for the symbol. Results shown are representative of 2 experiments.

3.5 *Discussion*

The data presented in this section show the elevation of calmodulin mRNA and functional protein levels in AtT20 cells that have been stably transfected with a recombinant mammalian expression vector (pcDNA3) containing a chicken calmodulin cDNA. However, CRF-stimulated ACTH secretion by these cells were apparently unchanged as compared to the secretion elicited by wild type AtT20 cells. This suggests that the constitutive over-expression system may not be the most appropriate system to address the issue of whether calmodulin may mediate early glucocorticoid feedback inhibition in AtT20 cells.

3.5.1 *Evaluation of the constitutive calmodulin over-expressing AtT20 system*

Calmodulin is a ubiquitous calcium-binding protein that has been shown to have wide ranging effects on physiological processes including cell growth, secretion and cell motility (Agell *et al.*, 1998; Hartwig *et al.*, 1992; Means *et al.*, 1991; Pritchard & Moody, 1986; Schulman, 1993). With regard to cell growth, it has been shown using anti-calmodulin drugs and genetic manipulation of calmodulin genes that calmodulin plays an important part in the regulation of nuclear functions like DNA synthesis and the onset and progression of mitosis *cf* (Bachs *et al.*, 1994). Given that calmodulin takes part in such fundamental processes required for maintaining cell viability, it seems likely that the level of biologically active calmodulin would be tightly regulated in the cell. In fact, it has been reported that over-expression of normal calmodulin led to cardiomyocyte hypertrophy in transgenic mice (Gruver *et al.*, 1993). Hence, one reason why elevated amounts of calmodulin mRNA and protein

could not be detected in most of the stably transfected calmodulin over-expressing AtT20 cell lines, may in part relate to the downregulation of the excess proteins [through ubiquitylation *cf* (Jennissen, 1995)] produced in these transfected AtT20 cell lines. Furthermore, recent studies have demonstrated that the reversible ubiquitylation of calmodulin by ubiquityl-calmodulin synthetase in the presence of Ca^{2+} strongly decreased the biological activity of calmodulin towards phosphorylase kinase (Laub & Jennissen, 1997; Laub & Jennissen, 1991). Therefore, although the excess calmodulin expressed in the transfected cell lines is not channeled to degradation by the 26S proteasome upon ubiquitylation (Varshavsky, 1997), its biological activity may be markedly reduced (Laub *et al.*, 1998).

Thus, from a physiological viewpoint, an inducible system that can be regulated to transiently overexpress calmodulin would be more favourable. Furthermore, such a system would simulate the early onset inhibition of glucocorticoid more accurately since *de novo* synthesis of mRNAs and proteins occur within the time scale (i.e. within 2h) of early onset glucocorticoid inhibition.

3.5.2 *Inducible expression systems for over-expressing calmodulin in AtT20 cells*

The artificial transcriptional regulatory system that uses the bacterial tetracycline operator/repressor first described by Gossen and Bujard (Gossen *et al.*, 1995) seems a suitable system to use. In particular, the rTet system which can drive the expression of calmodulin in AtT20 cells in response to varying concentrations of tetracycline or its derivative doxycycline would suit the purpose of mimicking glucocorticoid induction of new proteins well. In the initial attempts to assess the

feasibility of this inducible rTet system, the effect of doxycycline on ACTH secretion by wild type AtT20 cells was examined. Doxycycline was used because it was reportedly more potent (about 5-fold more than tetracycline) in driving gene expression in the rTet system.

Analysis of the characterisitics of ACTH secretion by AtT20 cells in the presence of doxycycline revealed that while basal secretion was not significantly modified, CRF-elicited secretion was significantly reduced when cells were treated with doxycycline for 30 min or 5 h (Figure 3.4). The duration of induction with doxycycline between 30 min and 5 h would most probably be appropriate for simulating the time scale for onset of early glucocorticoid inhibition. Therefore, any potential effect that the newly induced calmodulin may have on CRF-stimulated ACTH secretion will be masked by the inhibitory effect that doxycycline appears to exert on secretion. It is not clear why doxycycline inhibits ACTH secretion. One reason might be because Ca^{2+} has been reported to be important for the transport of tetracyclines (including doxycycline) in blood plasma (Berthon *et al.*, 1983; Jun & Lee, 1980; Lambs *et al.*, 1984). Hence, doxycycline may act as a calcium chelator and interfere with ACTH secretion which requires intracellular free Ca^{2+}. The tetracycline-regulated inducible expression system is thus not appropriate for addressing the role of calmodulin in early glucocorticoid inhibition of ACTH secretion by adenohypophysial corticotrophs.

Interestingly, a recent study (Schiller *et al.*, 1997) reported the use of AtT20 cells engineered to inducibly express the regulated endocrine-specific protein (RESP18)

using the same rTet inducible expression system. This inducible system appeared to work well for their study since they were able to demonstrate the existence of a novel signalling pathway from the secretory pathway lumen to the nucleus, using this system.

Other mammalian inducible expression systems have since become commercially available. Of note is the ecdysone-inducible expression system from Invitrogen, which drives gene expression with the analogues of an insect steroid hormone ecdysone. As Ponasterone A and Muristerone A (the ecdysone analogues) are reportedly inert to mammalian physiology, the ecdysone-inducible system may be useful for driving the transient over-expression of calmodulin in AtT20 cells and will not interfere with the assessment of any modulation of ACTH secretion in these cells.

4

INVESTIGATION OF THE MECHANISMS OF EARLYGLUCOCORTICOID INHIBITION IN RAT ANTERIOR PITUITARY PRIMARY CULTURES

4

INVESTIGATION OF THE MECHANISMS OF EARLYGLUCOCORTICOID INHIBITION IN RAT ANTERIOR PITUITARY PRIMARY CULTURES

4.1 Maintenance of the membrane potential in early glucocorticoid inhibition in corticotrophs

4.1.1 *Introduction*

CRF-stimulated ACTH release is mediated through cAMP-activated protein kinase enhancement of Ca^{2+} influx through voltage-gated Ca^{2+}-channels in the AtT20 mouse corticotroph tumour cell line (Luini *et al.*, 1985; Reisine *et al.*, 1986; Reisine *et al.*, 1985; Richardson, 1986). BK-channels, previously identified in AtT20 cells (Wong *et al.*, 1982), have been proposed as important negative feedback regulators of voltage-dependent Ca^{2+} influx (Twitchell *et al.*, 1997), thus important for restoring cellular excitability. Studies in AtT20 cells have shown that membrane depolarization antagonised glucocorticoid inhibition of stimulated ACTH release (Phillips & Tashjian Jr, 1982; Woods *et al.*, 1994). In addition, Ca^{2+}-channel activators (Antoni *et al.*, 1992; Pennington *et al.*, 1994) and K^{+}-channels inhibitors (Pennington *et al.*, 1994; Woods *et al.*, 1994), including BK-channel inhibitors (Shipston *et al.*, 1996), have also been demonstrated to antagonise glucocorticoid inhibition in AtT20 cells. These studies collectively indicated that early

glucocorticoid feedback inhibition targets the membrane potential in AtT20 cells. Hence, the objective of this section of the thesis was to investigate the importance of the maintenance of membrane potential in glucocorticoid inhibition of stimulated ACTH release in normal rat anterior pituitary corticotrophs. Furthermore, pharmacological tools were used in an effort to identify the underlying K^+-channels that may be involved in glucocorticoid inhibition of stimulated ACTH release. CRF at physiological concentrations and a membrane-permeant, non-metabolisable cAMP analogue, 8-(4-chlorophenylthio)adenosine-3',5'-cyclic-monophosphate (CPT-cAMP) were used as the primary secretagogue stimulus. CPT-cAMP mimics the increase in intracellular cAMP levels caused by CRF, but is not subject to the complex Ca^{2+}-dependent regulation of cAMP metabolism that is evident in corticotroph cells.

Part of the results presented in this section have been published as a full paper (Lim *et al.*, 1998).

Results

4.1.2 Eliciting membrane depolarization with common K^+-channel blockers and Ca^{2+}-channel activators

Experiments with AtT20 cells

4.1.2a *Response to CPT-cAMP and dexamethasone*

Concentration-dependent stimulation of ACTH release was evoked by CPT-cAMP (Figure 4.1) and the response plateaued at 1mM CPT-cAMP. Pretreatment for 2h with 100nM dexamethasone significantly reduced the increase in CPT-cAMP-stimulated ACTH secretion, basal hormone output remained unaltered (Figure 4.1).

4.1.2b *Effects of TEA and (-)BayK8644*

The ACTH response to 1mM CPT-cAMP could be enhanced further by the application of 5mM tetraethylammonium (TEA, a broad spectrum K^+-channel blocker) or the Ca^{2+}-channel activator (-)BayK8644 at 5μM (Figure 4.2a). Both of these compounds stimulated ACTH release when given alone and exhibited synergistic interaction with CPT-cAMP. Similar data were obtained with another L-channel activator (+)202-791 at 5μM (not shown).

The release of ACTH evoked by 1mM CPT-cAMP was inhibited by 65.8±8.9% (n=5) by 100nM dexamethasone, while basal secretion was not significantly altered (Figure 4.2a & b). The inhibition of ACTH secretion stimulated by a combination of

Figure 4.1

Concentration dependent increase of ACTH secretory response to CPT-cAMP in AtT20 cells

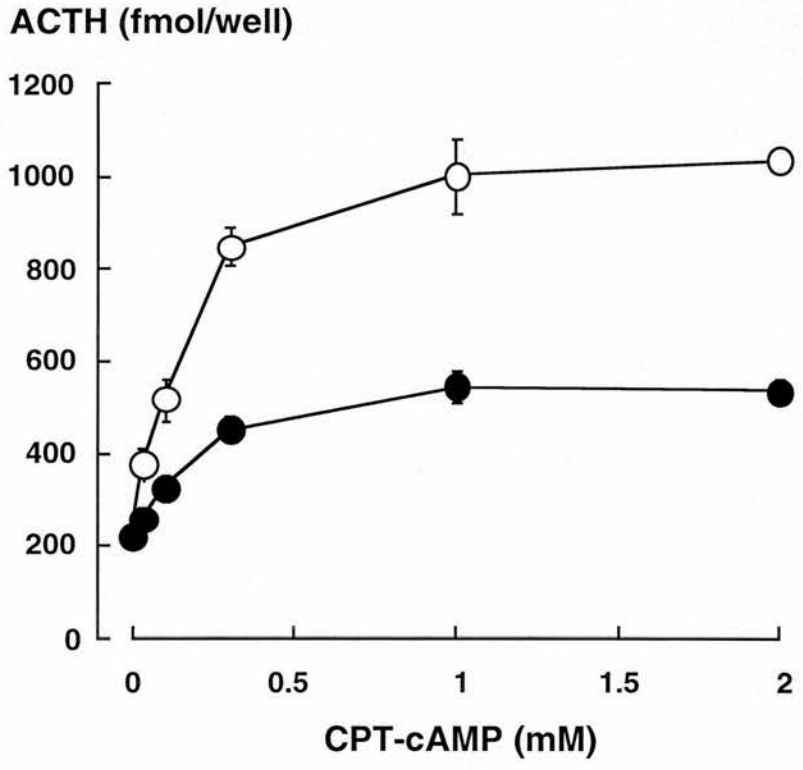

Figure 4.1: The effect of CPT-cAMP on ACTH secretion by AtT20 cells pretreated for 2h with vehicle (*empty circles*) or 100nM dexamethasone (*filled circles*). Means, the bars indicate SEM, n=5/group. Where error bars are not visible, the SEM bar is less than the space required for the symbol. Results shown are representative of 4 experiments.

Figure 4.2

Depolarising agents reduced dexamethasone inhibition of CPT-cAMP-induced ACTH release by AtT20 cells

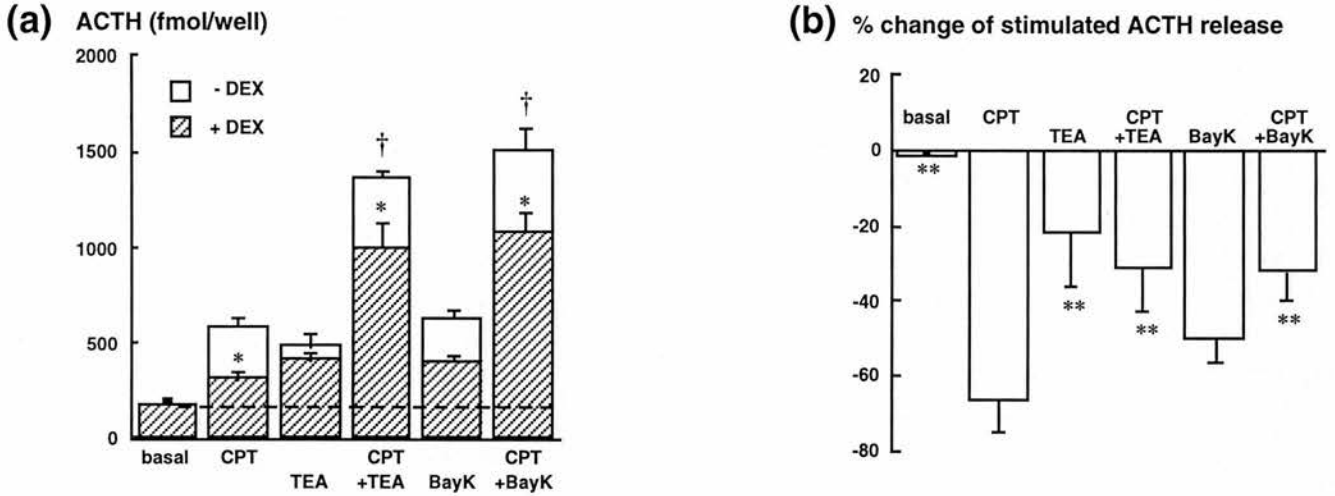

Figure 4.2: Effect of membrane depolarising agents — 5μM (-)BayK8644 (BayK) and 5mM tetraethylammonium (TEA) on ACTH secretion elicited by 1mM CPT-cAMP (CPT) after 2h preincubation with vehicle or 100nM dexamethasone in AtT20 cells. Columns are means, bars indicate SEM, n=4/group. In panel (a): The empty columns show ACTH release by vehicle treated cells and the hatched columns show release after treatment with dexamethasone. †$p<0.0209$ for the interaction between CPT alone and the respective depolarising agents alone, 2-way ANOVA. *$p<0.05$ when compared to corresponding vehicle-treated release, 1-way ANOVA followed by contrast of means. In panel (b): data are expressed as % change in the amount of ACTH secretion caused by dexamethasone when compared with the respective control groups shown in A. **$p<0.05$ when compared with CPT alone. 1-way-ANOVA followed by Dunnett's test. Results shown are representative of 2 experiments.

1mM CPT-cAMP and 5mM TEA or 1mM CPT-cAMP and 5μM (-)BayK8644 was significantly attenuated to 30.7±11.8% and 31.9±7.6% ($p<0.05$, n=5), respectively (Figure 4.2a & b). The combination of 1mM CPT-cAMP with 5μM (+)202-791 also significantly reduced the inhibition of the stimulated ACTH secretion by 100nM dexamethasone to 27±1.3% ($p<0.05$, n=4/group, 1-way-ANOVA, Dunnett's test).

As both (-)BayK8644 and TEA partially reversed dexamethasone inhibition of CPT-cAMP stimulated ACTH secretion, the combination of these compounds with CPT-cAMP was tested subsequently, using a submaximal concentration (0.1mM) of CPT-cAMP as well as 1mM CPT-cAMP. The ACTH secretion elicited by 0.1mM CPT-cAMP was significantly potentiated by 5μM (-)BayK8644 and 5mM TEA given together (Table 4.1). CPT-cAMP-induced ACTH secretion was inhibited by dexamethasone (1nM to 1μM) in a concentration dependent manner by up to 84% (Table 4.1). The combination of 5μM (-)BayK8644 and 5mM TEA elicited a 3-fold increase in ACTH secretion over basal levels and this response was not inhibited by dexamethasone (Table 4.1). The ACTH response elicited by the combination of 0.1mM CPT-cAMP, 5μM (-)BayK8644 and 5mM TEA was also fully resistant to inhibition by 1-1000 nM dexamethasone (Table 4.1).

Similar results were obtained by combining 1mM CPT-cAMP with 5mM TEA and 5μM (-)BayK8644. A summary of these data (Figure 4.3) shows that the extent of resistance to glucocorticoid inhibition was independent of the amplitude of stimulus-evoked ACTH secretion. For instance, although the ACTH release elicited by 5mM

Table 4.1

Modulation of dexamethasone inhibition of CPT-cAMP elicited ACTH release by TEA and (-)BayK8644 in AtT20 cells

	Dexamethasone (nM)				
	0	3	10	100	1000
Vehicle	608±63				
CPT	1122±136 *(100±26%)*	860±94 *(49±18%)*	795±57 *(36±11%)*	734±19 *(25±4%)*	690±22 *(16±4%)*
TEA+BayK	1883±65 *(100±5%)*	1973±125 *(107±10%)* †	1767±57 *(91±4%)* †	1783±84 *(92±7%)* †	1743±167 *(89±13%)* †
CPT+TEA+BayK	2919±93 * *(100±4%)*	2912±126 *(100±5%)* †	2593±170 *(86±7%)* †	2626±62 *(87±3%)* †	3016±161 *(104±7%)* †

Table 4.1: Characterisation of the ACTH secretory response to 0.1mM CPT-cAMP (CPT), 5mM TEA and (-)BayK8644 (5μM) (BayK) in the presence of increasing dexamethasone concentrations in AtT20 cells. Data show ACTH release expressed in fmol/well and represent mean±SEM of 4 replicates per treatment group. Values in parentheses represent percentages of control ACTH release (mean±SEM), as defined in section 2.7.2. *$p<0.02$ for the interaction between CPT and TEA/BayK in the absence of dexamethasone pretreatment, 2-way ANOVA. Statistical analysis of the effect of dexamethasone was carried out on the data expressed as percentages (values shown in parentheses) of the respective stimulus-evoked release, †$p<0.05$ when compared to the corresponding CPT group, 1-way ANOVA followed by Dunnett's test. Results shown are representative of 2 experiments.

Figure 4.3

Size of the ACTH secretory response failed to predict the degree of dexamethasone inhibition in AtT20 cells

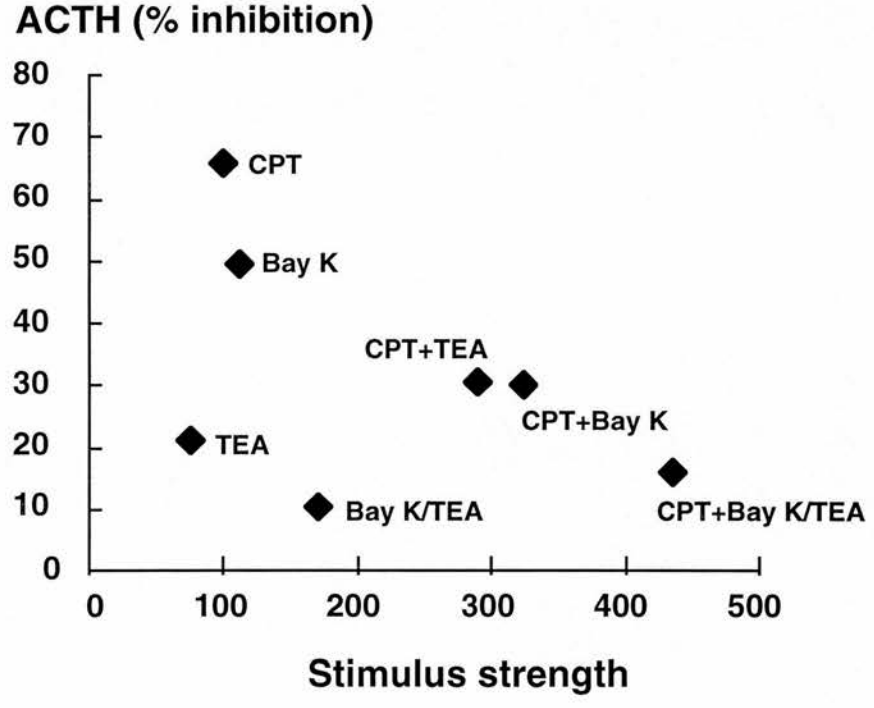

Figure 4.3: Data were plotted as stimulus strength *vs.* % inhibition of ACTH release, where stimulus strength is the size of the evoked release of ACTH expressed as % of the response to 1mM CPT-cAMP run in the same experiment, and % inhibition of ACTH release is the degree of inhibition of the ACTH response to the respective stimulus in cells preincubated with 100nM dexamethasone, expressed as percentage of the control (i.e. no dexamethasone) response (see section 2.3.1). Data are means n=4/group. Symbols: CPT—1mM CPT-cAMP; BayK—5μM (-) BayK8644; TEA—5mM tetraethylammonium; BayK/TEA — combination of 5μM (-)BayK8644 and 5mM TEA; + sign indicates that two stimuli were applied together. Results shown are representative of 2 experiments.

TEA or 1mM CPT-cAMP alone were similar, the ACTH response to 5mM TEA was much more resistant to dexamethasone inhibition than the response to 1mM CPT-cAMP.

Taken together, these results are in agreement with previous studies of CRF-induced ACTH release in AtT20 cells, which have shown that early glucocorticoid inhibition is effectively counteracted by depolarization of the membrane potential (Woods *et al.*, 1994) and that TEA sensitive K^+-channels underlie the inhibitory action of corticosteroids (Shipston *et al.*, 1996).

Experiments with primary cultures of rat anterior pituitary

4.1.2c *Response to CPT-cAMP*

The time-course of the ACTH response to CPT-cAMP was close to linear over 4h (Figure 4.4a). The concentration-response relationship between CPT-cAMP and ACTH release was similar at 60,120 and 240 min, in that 0.1 mM CPT -cAMP elicited close to maximal stimulation of ACTH release (Figure 4.4b). This was not the case at 30 min, where 1mM CPT-cAMP was significantly more effective than 0.1mM CPT-cAMP. All subsequent experiments were terminated at the 60 min time-point.

4.1.2d *Lack of effect of TEA and (-)BayK8644 on glucocorticoid inhibition of ACTH release*

Corticosterone (100nM) reduced the ACTH response to 0.1mM CPT-cAMP by 74.8±10.8% (n=5) and this was not modified in the presence of TEA (5-20mM) or (-)BayK8644 (5μM). Similar findings were obtained with 30nM dexamethasone. Combination of 5mM TEA and (-)BayK8644 (5μM) produced a significant enhancement of the ACTH releasing effect of CPT-cAMP (0.3mM) (in fmol/well---- Basal: 34±6; CPT-cAMP: 125±5; TEA+BayK: 60±6; CPT-cAMP+TEA+BayK: 204±14, n=5-7/group, $p<0.05$ for the interaction between CPT and TEA+BayK, 2-way-ANOVA) but failed to modify the inhibition by 100nM dexamethasone (% inhibition of CPT-cAMP *vs* CPT-cAMP+TEA+BayK: 63±7% *vs* 66±6%, n=4).

Qualitatively similar data were obtained using 0.1nM CRF as the primary secretagogue (summarized in Table 4.2). TEA (10mM), (-)BayK8644 (5μM) or a

combination of both drugs produced a synergistic enhancement of the ACTH response to 0.1nM CRF. Corticosterone (100nM) inhibition of 0.1nM CRF-evoked ACTH release was unaltered in the presence of 10mM TEA, (-)BayK8644 (5μM) or a combination of both drugs (Table 4.2).

These data indicate that in contrast to AtT20 cells, TEA-sensitive ion-channels are not fundamentally important for corticosteroid inhibition of ACTH secretion in primary cultures of rat anterior pituitary cells.

4.1.2e *Other common K^+-channel blockers also failed to modify corticosterone inhibition*

Effects of apamin and 4-aminopyridine (4-AP), inhibitors of small conductance Ca^{2+}-activated K^+-channels (SK-channels) and transient A-type K^+-channels respectively, on corticosterone inhibition were also investigated. CRF (0.1nM)-stimulated ACTH release was not significantly modified by pretreatment with 100nM apamin or 1mM 4-AP (in fmol/well----Basal: 78.1±2.8; CRF: 182.2±16.3; CRF+apamin: 196.8±24.2; CRF+4-AP: 212.0±11.5, n=4-6/group). Corticosterone (100nM) reduced 0.1nM CRF-induced ACTH response by 81.0±6.1% (n=6) and was not significantly modified by 100nM apamin or 1mM 4-AP (% inhibition----CRF+apamin: 73.0±6.4, n=4; CRF+4-AP: 70.7±4.5, n=4/group).

Figure 4.4

Effect of CPT-cAMP on ACTH secretion by primary cultures of rat anterior pituitary cells

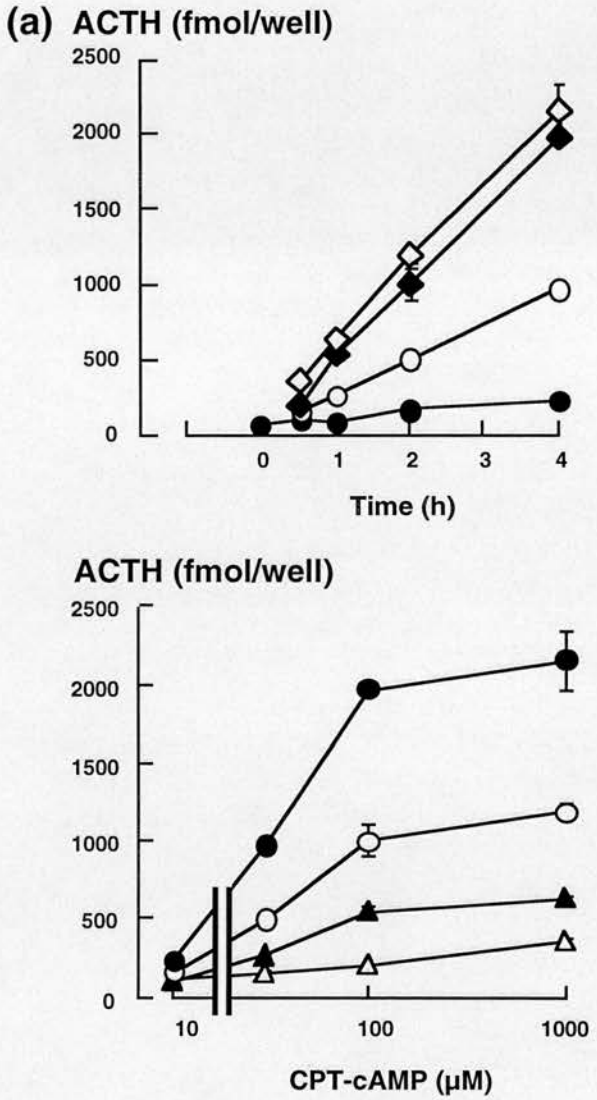

Figure 4.4: (a) Time-course of hormone release in cells treated with vehicle (*filled circles),* 30μM CPT-cAMP (*empty circles*), 0.1mM CPT-cAMP (*filled diamonds*) or 1mM CPT-cAMP (*empty diamonds*). (b) Concentration-dependent stimulation of ACTH secretion by CPT-cAMP at 30min (*empty triangles*), 1 h (*filled triangles*), 2 h (*empty circles*) and 4 h (*filled circles*) of incubation. Data are means±SEM, n=4/group. Where error bars are not visible, the SEM bar is less than the space required for the symbol. Results shown are representative of 2 experiments.

Table 4.2

Inhibition of CRF-stimulated ACTH secretion by corticosterone was unaltered by TEA and BayK in normal rat corticotrophs

	Veh	CORT
Veh	41.65 ± 4.20	
CRF	116.56 ± 5.44 *(100 ± 7.26 %)*	66.73 ± 4.04 *(33.48 ± 5.39 %)*
TEA	37.95 ± 2.62	
BayK	40.28 ± 3.04	
TEA+BayK	42.94 ± 2.03	
CRF+TEA	154.75 ± 9.18 † *(100 ± 8.12 %)*	73.49 ± 4.04 *(28.15 ± 3.57 %)*
CRF+BayK	143.37 ± 7.14 † *(100 ± 7.02 %)*	73.82 ± 4.46 *(31.63 ± 4.38 %)*
CRF+TEA+BayK	196.84 ± 16.00 † *(100 ± 10.31 %)*	104.07 ± 3.10 *(40.22 ± 2.00 %)*

Table 4.2: Effect of 10mM TEA and 5μM (-)BayK 8644 (BayK) on 100nM corticosterone (CORT) inhibition of 0.1nM CRF-stimulated ACTH secretion. Data show ACTH release expressed in fmol/well and represent means ± SEM (n=4/group). Values in parentheses represent percentages of control ACTH release (means±SEM, n=4/group), as defined in section 2.7.2. † $p<0.05$ for the interaction between CRF and TEA, BayK or TEA/BayK in the absence of corticosterone pretreatment, 2-way-ANOVA. Statistical analysis of the effect of CORT, carried out on the data expressed as percentages (values in parentheses) of the respective stimulus-evoked release, showed no statistical difference (1-way-ANOVA). Results shown are representative of 2 experiments.

4.1.3 Characteristics of CRF-stimulated ACTH release in the presence of depolarizing concentration of KCl in cultured rat anterior pituitary cells

4.1.3a *Depolarization with 40mM KCl counteracts glucocorticoid inhibition of ACTH release*

The ACTH responses to 0.1mM CPT-cAMP and 40mM KCl are shown in Table 4.3. KCl (40mM) synergised with 0.1mM CPT-cAMP to stimulate ACTH release, furthermore, it produced a marked reduction in the inhibitory effect of dexamethasone (Table 4.3). Dexamethasone (10nM) inhibited the ACTH response to 0.1mM CPT-cAMP and 40mM KCl by only 44±12% (n=4), while that evoked by 0.1mM CPT-cAMP was completely blocked. Similar results were also obtained using the naturally occurring corticosteroid corticosterone (Table 4.3)

The ACTH responses to 0.1nM CRF and 40mM KCl were also examined (Figure 4.5a). In contrast to the synergistic effects of combining 0.1mM CPT-cAMP and 40mM KCl, the ACTH release elicited by the combination of 0.1nM CRF and 40mM KCl was additive in 5 out of 7 experiments (synergism was observed only in 2 experiments). Prevention of Ca^{2+} entry by 0.1mM cadmium completely blocked ACTH release elicited by CRF alone or in combination with KCl (see Table 4.5). Corticosterone (10-1000nM) inhibition of the ACTH response to 0.1nM CRF and 40mM KCl was markedly reduced (Figure 4.5b).

The data above demonstrate that depolarization of the membrane potential with KCl markedly reduces the early inhibitory effect of glucocorticoids in normal rat corticotrophs.

4.1.3b *Blockers of mRNA synthesis abolished early glucocorticoid inhibition*

The inhibition of 0.1mM CPT-cAMP stimulated ACTH secretion by 10nM dexamethasone was eliminated in the presence of 5,6-dichloro-furanosyl-benzimidazole riboside (DRB), an adenosine analogue and inhibitor of heteronuclear RNA synthesis (Egyhazi *et al.*, 1982) (Figure 4.6a). In the presence of 40mM KCl, DRB suppressed ACTH secretion elicited by 0.1mM CPT-cAMP but no further change was observed after treatment with dexamethasone (Figure 4.6b). Therefore, the residual inhibition by corticosteroids observed in the presence of 40mM KCl and 0.1mM CPT-cAMP is likely to be due to glucocorticoid blockade of this DRB sensitive mechanism.

Table 4.3

Counteraction of glucocorticoid inhibition of the ACTH secretory response by KCl in cultured rat anterior pituitary cells

	Corticosterone (nM)				
	0	3	10	100	1000
Vehicle	100.4±11.0				
CPT	163.8±6.9 *(100±11%)*	150.8±8.1 *(80±13%)*	132.6±15.2 *(51±24%)*	86.4±2.1 *(-22±3%)*	80.3±3.0 *(-31±5%)*
KCl	125.5±2.9				
CPT+KCl	284.8±4.1 * *(100±2%)*	269.8±16.3 *(92±9%)*	260.8±7.6 *(87±4%)* †	221.9±23.1 *(66±13%)* †	216.3±22.7 *(63±12%)* †

	Dexamethasone (nM)			
	0	3	10	100
Vehicle	66.1±7.0			
CPT	112.8±5.3 *(100±11%)*	62.8±2.5 *(-7±5%)*	54.5±3.3 *(-25±7%)*	52.0±3.1 *(-30±7%)*
KCl	127.6±14.4			
CPT+KCl	280.6±27.2 # *(100±13%)*	192.2±31.8 *(59±15%)* †	187.2±26.1 *(56±12%)* †	165.2±11.8 *(46±6%)* †

Table 4.3: Data show ACTH release expressed in fmol/well and represent mean±SEM of 4 replicates per treatment group. Values in parentheses represent percentages of control ACTH release (mean±SEM), as defined in section 2.7.2. *$p<0.0001$ and #$p<0.004$ for the interaction between CPT-cAMP and KCl in the absence of glucocorticoid pretreatment, 2-way ANOVA. Statistical analysis of the effect of glucocorticoids was carried out on the data expressed as percentages (values shown in the parentheses) of the respective stimulus-evoked release, †$p<0.05$ when compared to the corresponding CPT group, 1-way ANOVA followed by contrast of means. Results shown are representative of 2 and 3 experiments for the corticosterone and dexamethasone dose responses respectively.

Figure 4.5

Effect of 40mM KCl on CRF-induced ACTH release by cultured rat anterior pituitary cells

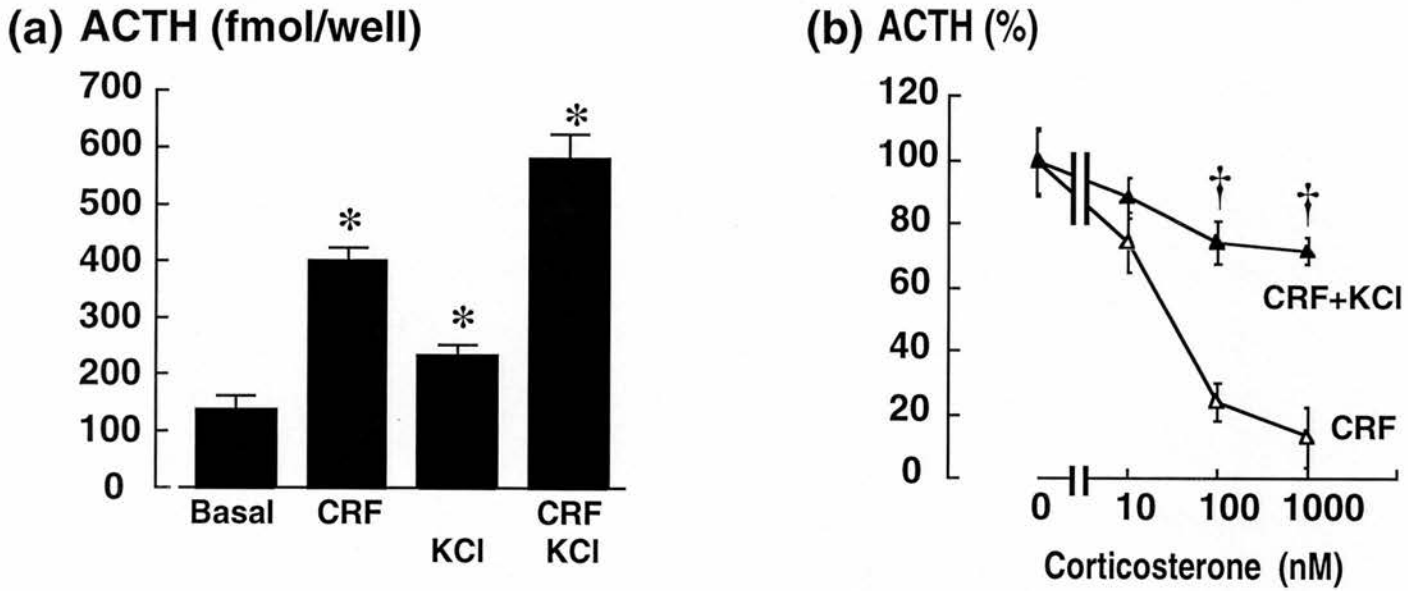

Figure 4.5: (a) ACTH response to 0.1nM CRF and 40mM KCl. Solid columns are means, bars indicate SEM, n=4/group. *P<0.05 when compared to basal ACTH release, 1-way ANOVA followed by Newman-Keuls test. (b) Concentration dependent inhibition of 0.1nM CRF-evoked ACTH release by corticosterone in the absence and presence of 40mM KCl. Data are expressed as percent of control ACTH release (as defined in section 2.3.1) and represent means±SEM. †P<0.05 when compared to corresponding CRF group, 1-way ANOVA followed by contrast of means. Results shown are representative of 3 experiments.

Figure 4.6

Effect of the RNA synthesis inhibitor 5,6-dichloro-furanosyl-benzimidazole riboside (DRB) on ACTH secretion in cultured rat anterior pituitary cells

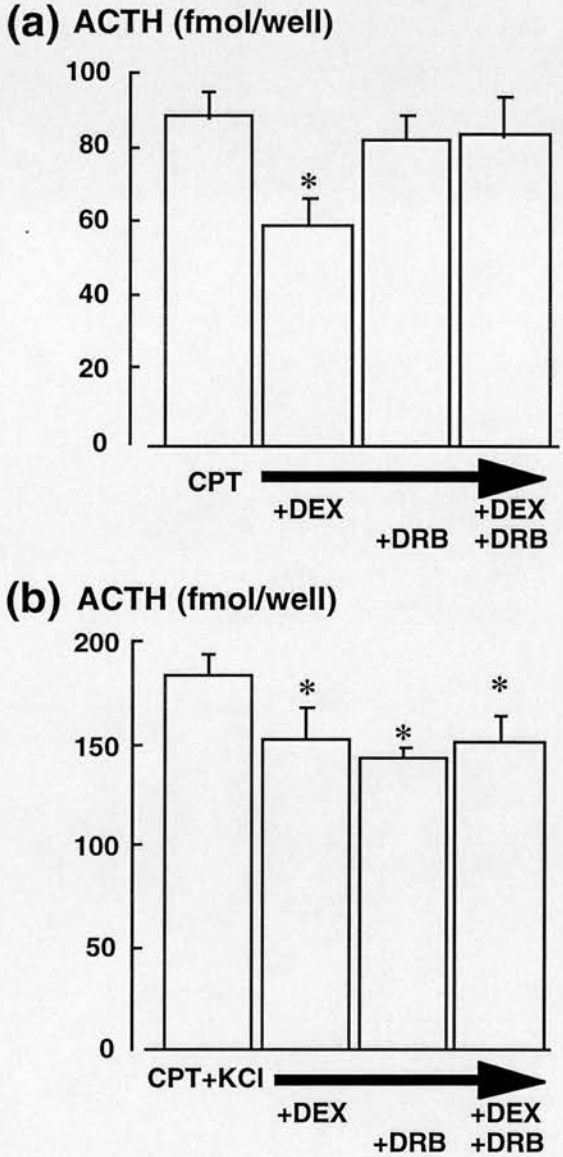

Figure 4.6: DRB (100μM) and 100nM dexamethasone (DEX) were applied 2h before challenging the cells with (A) 0.1mM CPT-cAMP or (B) 0.1mM CPT-cAMP and 40mM KCl for 60 min. Basal ACTH release was 44±3 fmol/well and was not altered by DRB or dexamethasone. Columns are means, bars indicate SEM, n=5/group. * $P<0.05$ when compared with control group receiving vehicle only. 1-way-ANOVA followed by Newman-Keuls test. Results shown are representative of 4 experiments.

4.1.4 Role of BK-channels in glucocorticoid inhibition of stimulated ACTH response

Experiments with AtT20 cells

4.1.4a *Charybdotoxin reduced dexamethasone inhibition of CPT-cAMP-evoked ACTH release*

A potent inhibitor of BK-channels, charybdotoxin (ChTx), was used to verify the involvement of BK-channels in the inhibitory action of glucocorticoid in AtT20 cells. Charybdotoxin (100nM) had no significant effect on the ACTH response elicited by 0.1mM CPT-cAMP (in fmol/well----Basal: 165 ± 9.8; CPT-cAMP: 507 ± 45.9; ChTx: 217 ± 19.7; CPT-cAMP+ChTx: 588 ± 65.0, n=4/group). Dexamethasone (30nM) inhibition of 0.1mM CPT-cAMP elicited ACTH release was markedly reduced in the presence of 100nM charybdotoxin (% inhibition of CPT-cAMP *vs* CPT-cAMP+ChTx: 98.1 ± 9.8% *vs* 49.5 ± 7.6%, n=4, $p<0.05$, Students two-tailed, unpaired t-test).

These data are in agreement with results from a previous study (Shipston *et al.*, 1996) that dexamethasone inhibition of stimulated ACTH release are antagonised by inhibitors of BK-channels in AtT20 cells. Therefore, BK-channels appear to play a pivotal role in corticosteroid inhibition in AtT20 cells.

Experiments with rat anterior pituitary primary cultures

4.1.4b *Charybdotoxin failed to modify glucocorticoid inhibition of stimulated ACTH release*

Table 4.4 summarizes the effect of 100nM charybdotoxin on 0.1mM CPT-cAMP and 0.1nM CRF elicited ACTH release in the absence and presence of 100nM corticosterone in normal rat corticotrophs. As was observed in AtT20 cells, the ACTH response to 0.1mM CPT-cAMP or 0.1nM CRF was not significantly modified by 100nM charybdotoxin. Notably, the ACTH release elicited by the combination of 100nM charybdotoxin with 0.1nM CRF or 0.1mM CPT was inhibited by 100nM corticosterone to the same extent as in the absence of charybdotoxin (Table 4.4).

In sum, these data together with that in Section 4.1.2d suggest that BK-channels have no apparent role in corticosteroid inhibition in normal rat corticotrophs.

Table 4.4

Lack of effect of charybdotoxin on corticosterone inhibition of secretagogue-evoked ACTH response in normal rat coticotrophs

	Veh	CORT
Veh	23.50 ± 1.91	
ChTx	27.69 ± 3.27	
CPT	47.83 ± 2.33 *(100 ± 9.58 %)*	28.65 ± 4.28 *(21.18 ± 17.58 %)*
CPT+ChTx	50.10 ± 4.04 *(100 ± 15.18 %)*	29.81 ± 3.56 *(23.73 ± 13.37 %)*
CRF	87.38 ± 13.16 *(100 ± 20.59 %)*	39.48 ± 2.83 *(25.01 ± 4.44 %)*
CRF+ChTx	97.02 ± 5.15 *(100 ± 7.01 %)*	49. 71 ± 7.60 *(35.65 ± 10.34 %)*

Table 4.4: Effect of 100nM charybdotoxin (ChTx) on 100nM corticosterone (CORT) inhibition of 0.1mM CPT-cAMP (CPT) or 0.1nM CRF-stimulated ACTH release. Data show ACTH release expressed in fmol/well and represent means ± SEM (n=4/group). Values in parentheses represent percentages of control ACTH release (means±SEM, n=4/group), as defined in section 2.7.2. Statistical analysis of the effect of CORT, carried out on the data expressed as percentages (values in parentheses) of the respective stimulus-evoked release, showed no statistical difference (1-way-ANOVA). Results shown are representative of 2 experiments.

4.1.5 Analysis of the involvement of novel K^+-channels in corticosteroid inhibition in rat anterior pituitary primary cultures

In order to probe further the K^+-channels that may have a role in corticosteroid inhibition, inhibitors of other less conventional K^+-channels were tested for any potential effect on corticosteroid inhibition. Recent molecular, pharmacological and electrophysiological studies have revealed novel K^+-channels responsible for the repolarization of the cardiac action potential (Sanguinetti & Jurkiewicz, 1990). These are the human ether-a-go-go related-gene (HERG)-channels and the KvLQT1/IsK channels (see section 4.1.5c). The induction of similar channel proteins have been linked to corticosteroids (Attali *et al.*, 1995). In this study (Attali *et al.*, 1995), a K^+-specific channel activity (which biophysical, pharmacological, and regulatory characteristics are very similar to those reported before for IsK) was expressed in Xenopus laevis oocytes. In rat GH3 anterior pituitary cells, pharmacological and kinetic evidence has indicated that thyrotrophin-releasing hormone regulates a HERG-like K^+-current that is important for maintaining cellular excitability in these cells (Barros *et al.*, 1997). Furthermore, RT-PCR analysis has revealed that mRNA of the rat homologue of HERG is present in GH3/B6 cells (Bauer *et al.*, 1998). It is therefore conceivable that HERG-type K^+-channels may also be expressed in rat adenohypophysial corticotrophs. Consequently, the effects of clofilium (a KvLQT1/IsK-type K^+-channel blocker,) and astemizole (an antihistamine with anti-HERG-type K^+-channel properties) on corticosteroid inhibition of stimulated ACTH secretion were examined.

4.1.5a *Corticosterone inhibition of CRF-induced ACTH response was reduced by clofilium and astemizole*

The effects of clofilium and astemizole on corticosterone inhibition of 0.1nM CRF-stimulated ACTH release were compared to that of 40mM KCl (Figure 4.7). Figure 4.7a shows the ACTH release induced by 0.1nM CRF alone and in combination with 10μM clofilium, 10μM astemizole or 40mM KCl before and after 100nM corticosterone pretreatment. Inhibitory effect of 100nM corticosterone on 0.1nM CRF-stimulated ACTH release was significantly attenuated in the presence of 10μM clofilium or 10μM astemizole, similar to the antagonism of corticosterone inhibition seen in the presence of 40mM KCl (Figure 4.7b).

Since clofilium and astemizole appear to reduce corticosterone inhibition, the effects of these compounds were analyzed in further detail. The dose response analysis of clofilium (Figure 4.8a) and astemizole (Figure 4.8b) indicated that at 10μM, the inhibitors significantly increased the ACTH response to 0.1nM CRF in the presence of 100nM corticosterone pretreatment. This concentration (10μM) was also the maximal non-toxic concentration that could be used for ACTH secretion studies in rat anterior pituitary cultures. Cells started to detach from the culture dishes when these inhibitors were used at concentrations higher than 10μM. Clofilium (10μM) had no significant synergistic effect on the ACTH response to 0.1nM CRF (Figure 4.9a) but shifts the curve for the concentration dependent inhibition of 0.1nM CRF-stimulated ACTH release by corticosterone (10nM-100nM) to the right by an order of magnitude (Figure 4.9b). Astemizole (10μM) had no significant effect on 0.1nM

CRF-induced ACTH release (Figure 4.10a) and markedly reduced the efficiency of corticosterone (30nM-1000nM) inhibition of 0.1nM CRF-induced ACTH release (Figure 4.10b).

In sum, the data suggested that clofilium- and astemizole-sensitive ion channels, in particular, KvLQT1/IsK-type and/or HERG-type K^+-channels may be involved in corticosteroid inhibition in normal rat corticotrophs.

Figure 4.7

Corticosterone inhibition of CRF-evoked ACTH release was similarly antagonised in the presence of clofilium, astemizole and KCl in cultured rat anterior pituitary cells

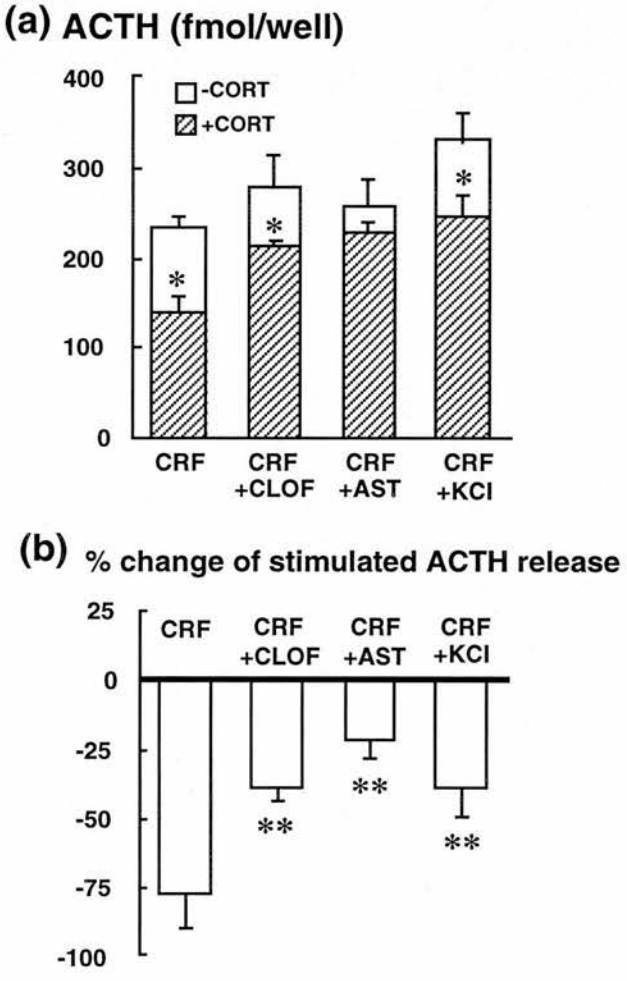

Figure 4.7: Effect of 10μM clofilium (CLOF), 10μM astemizole (AST) and 40mM KCl on ACTH release elicited by 0.1nM CRF after pretreatment with vehicle or 100nM corticosterone (CORT). Columns are means, bars indicate SEM, n=4-6/group. (a) The empty columns show ACTH release by vehicle treated cells and the hatched columns show release after treatment with 100nM corticosterone. Basal ACTH release is 110.3±10.6 fmol/well. *P<0.05 when compared to corresponding vehicle treated release, 1-way ANOVA followed by contrast of means. (b) Data are expressed as % change in the amount of ACTH release caused by corticosterone when compared with the respective control groups shown in (a). **P<0.05 when compared with CRF alone, 1-way ANOVA followed by Dunnett's test. Results shown are representative of 2 experiments.

Figure 4.8

Effects of clofilium and astemizole on stimulated ACTH response in cultured rat anterior pituitary cells

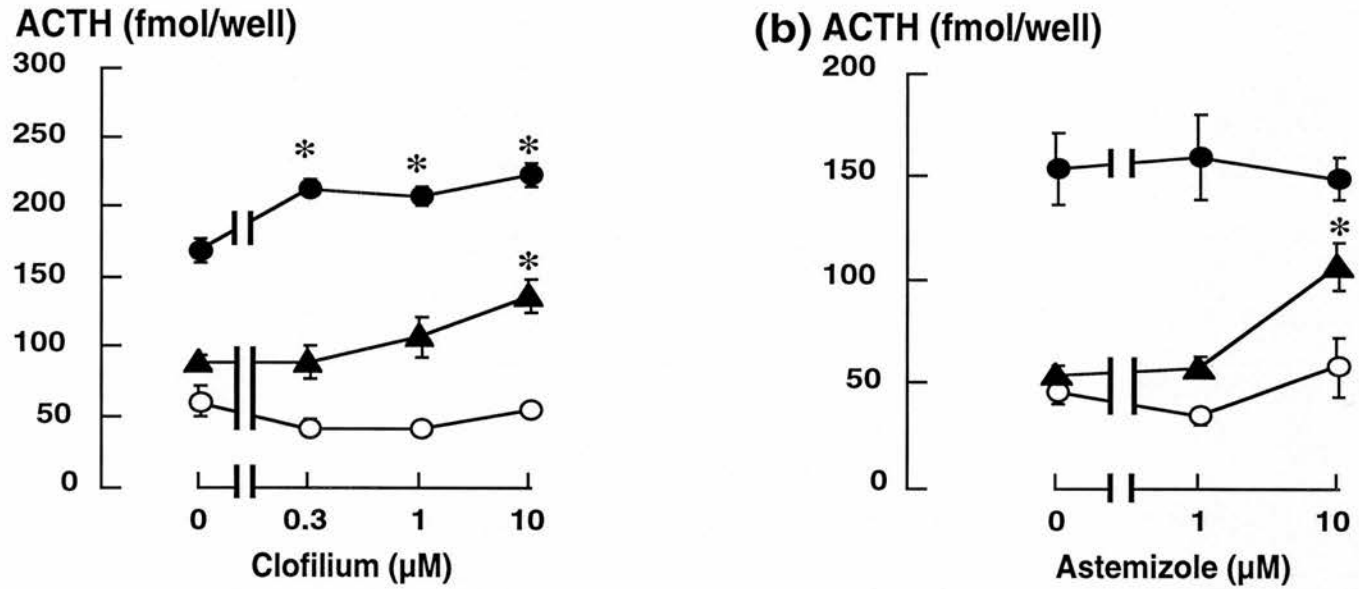

Figure 4.8: Effects of (a) clofilium and (b) astemizole on basal *(empty circles)* or 0.1nM CRF-evoked ACTH release in the absence *(filled circles)* and presence *(filled triangles)* of 100nM corticosterone (CORT) pretreatment. K^+-channel blockers were applied as described in section 2.3. Data are means±SEM, n=3-7/group. *P<0.05 when compared to corresponding stimuli in the absence of clofilium or astemizole. Where error bars are not visible, the SEM bar is less than the space required for the symbol. Results shown are representative of 3 and 2 experiments for (a) and (b) respectively.

Figure 4.9

Efficiency of corticosterone inhibiton of CRF-stimulated ACTH release was reduced by clofilium in primary cultures of rat anterior pituitary cells

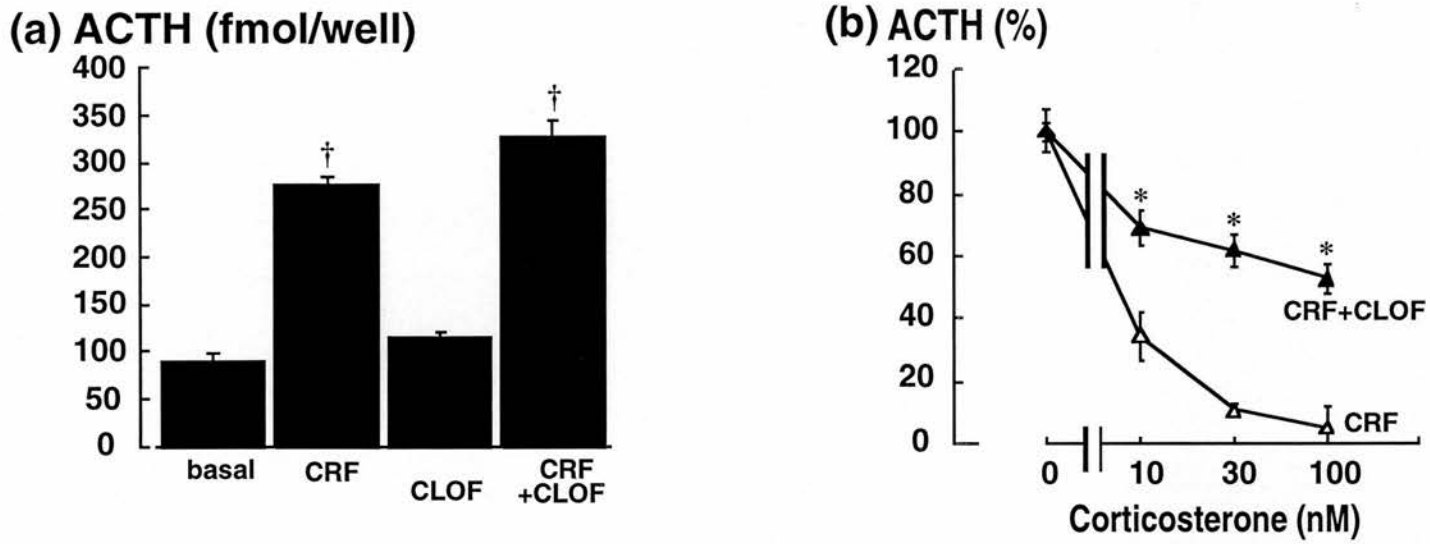

Figure 4.9: (a) ACTH response to 0.1nM CRF (CRF) and 10μM clofilium (CLOF). Solid columns are means, bars indicate SEM, n=4-5/group. (b) Corticosterone inhibition of 0.1nM CRF-evoked ACTH release in the absence and presence of 10μM clofilium pretreatment (application protocol defined in section 2.2). Data are expressed as percent of control ACTH release (as defined in section 2.3.1) and represent means±SEM, n=4-5/group. †P<0.05 when compared to basal, and also between the 2 groups, 1-way ANOVA followed by Newman-Keuls test. *P<0.05 when compared to corresponding CRF group, 1-way ANOVA followed by contrast of means. Results shown are representative of 3 experiments.

Figure 4.10

Efficiency of corticosterone inhibition of CRF-stimulated ACTH secretion was also reduced by astemizole

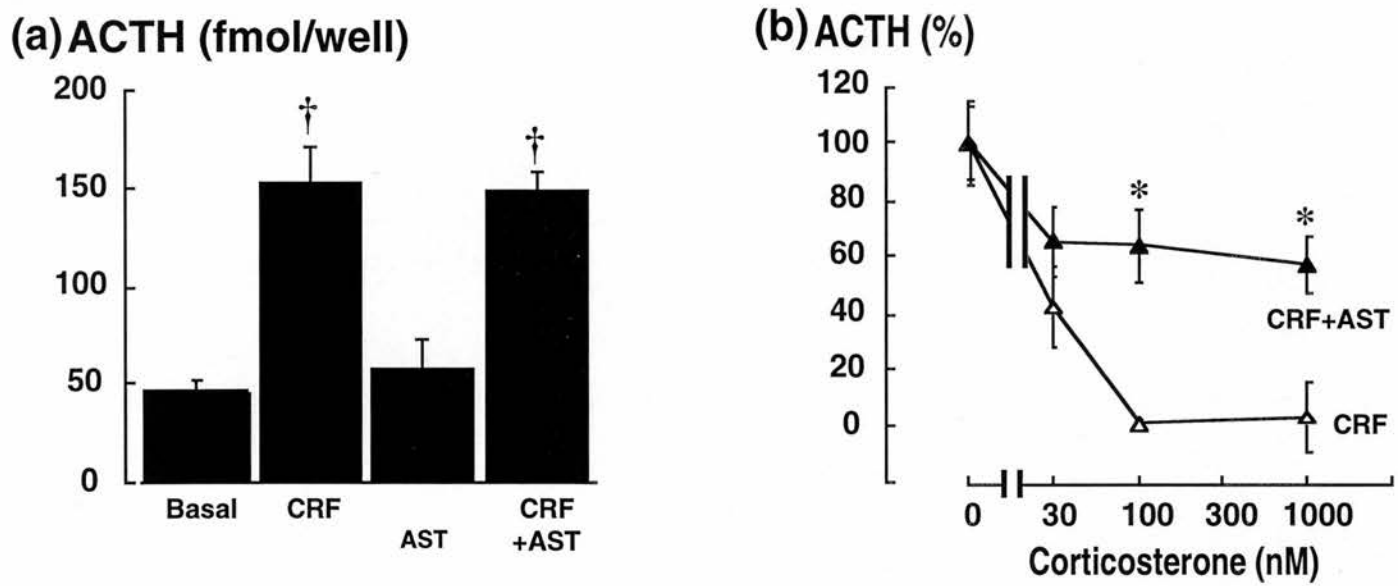

Figure 4.10: (a) ACTH response to 0.1nM CRF(CRF) and 10μM astemizole(AST). Solid columns are means, bars indicate SEM, n=3-5/group. (b) Concentration dependent inhibition of 0.1nM CRF-evoked ACTH release by corticosterone in the absence and presence of 10μM astemizole pretreatment (application protocol defined in section 2.2). Data are expressed as percent of control ACTH release (defined in section 2.3.1) and represent means±SEM, n=4-5/group. †P<0.05 when compared to basal, 1-way ANOVA followed by Newnam-Keuls test. *P<0.05 when compared to corresponding CRF group, 1-way ANOVA followed by contrast of means. Results shown are representative of 4 experiments.

4.1.5b *Effect of cadmium on CRF-induced ACTH release and cAMP accumulation in the presence of clofilium or astemizole*

To investigate whether ACTH secretory mechanisms (in particular, requirement of $[Ca^{2+}]_i$ increase for secretion) normally utilized by CRF were modified in the presence of clofilium and astemizole, the effect of a commonly used inorganic divalent ion Ca^{2+}-channel blocker Cd^{2+} was tested.

CRF (0.1nM)-elicited ACTH release was completely abolished by 0.1mM $CdSO_4$ (Cd^{2+})(Table 4.5). The ACTH response induced by the combination of 0.1nM CRF and 10μM clofilium or 10μM astemizole was significantly reduced but not abolished by 0.1mM Cd^{2+} added 30 min before and during the stimulation with the secretagogues (Table 4.5). Cd^{2+} (0.1mM) inhibited the ACTH response to 0.1nM CRF/10μM clofilium and 0.1nM CRF/10μM astemizole by 58.7±6.4% (n=4) and 62.5±4.7% (n=2) respectively. Since 100nM corticosterone blocked 40% and 25% of the ACTH response to 0.1nM CRF in the presence of 10μM clofilium and 10μM astemizole respectively (see Figure 4.7b), it appears that corticosterone blocked all of the Cd^{2+}-sensitive ACTH response to 0.1nM CRF in the presence of these drugs.

Cd^{2+} (0.1mM) pretreatment had no effect on basal cAMP accumulation but significantly enhanced the cAMP response elicited by 0.1nM CRF (Figure 4.11).

These results indicate that ACTH secretion elicited by a combination of CRF with clofilium or astemizole is largely dependent on $[Ca^{2+}]_I$, therefore largely similar to

the mechanism of CRF-induced release. Furthermore, the suppression of ACTH secretion by Cd^{2+} is not caused by a suppression of cAMP levels. Nonetheless, distinct mechanism(s) could be mediating ACTH secretion elicited by CRF in the presence of clofilium or astemizole given that Cd^{2+} completely blocked CRF-induced ACTH release but only partially blocked CRF/drug-induced ACTH release.

Table 4.5

Blocking Ca^{2+} entry with cadmium reduced secretagogue-evoked ACTH response in normal rat corticotrophs

	Veh	Cd
Veh	108.36 ± 3.28 (4)	117.72 ± 10.67 (3)
CRF	241.99 ± 10.95 (4)	132.55 ± 5.69 (4)
CRF+CLOF	243.70 ± 18.42 (4)	164.22 ± 8.71 (4)*
CRF+AST	270.57 ± 13.59 (2)	169.24 ±7.70 (2)*
CRF+KCl	338.58 ± 10.83 (3)	142.51 ± 15.86 (3)

Table 4.5: Effect of 0.1mM $CdSO_4$ (Cd^{2+}) on 0.1nM CRF-stimulated ACTH release alone or in combination with 10μM clofilium (CLOF), 10μM astemizole (AST) or 40mM KCl. Data show ACTH release expressed in fmol/well and represent means±SEM. Cells were pretreated for 30 min with Cd^{2+}, CLOF and AST accordingly before being challenged with the various secretagogues. *$P<0.05$ when compared to cells treated only with Cd^{2+} alone, 1-way-ANOVA followed by Student-Newman-Keuls' test. Values shown in parentheses indicate the number of experiments that mean values were taken from.

Figure 4.11

cAMP accumulation elicited by CRF is enhanced by Cd^{2+} in primary cultures of rat anterior pituitary cells

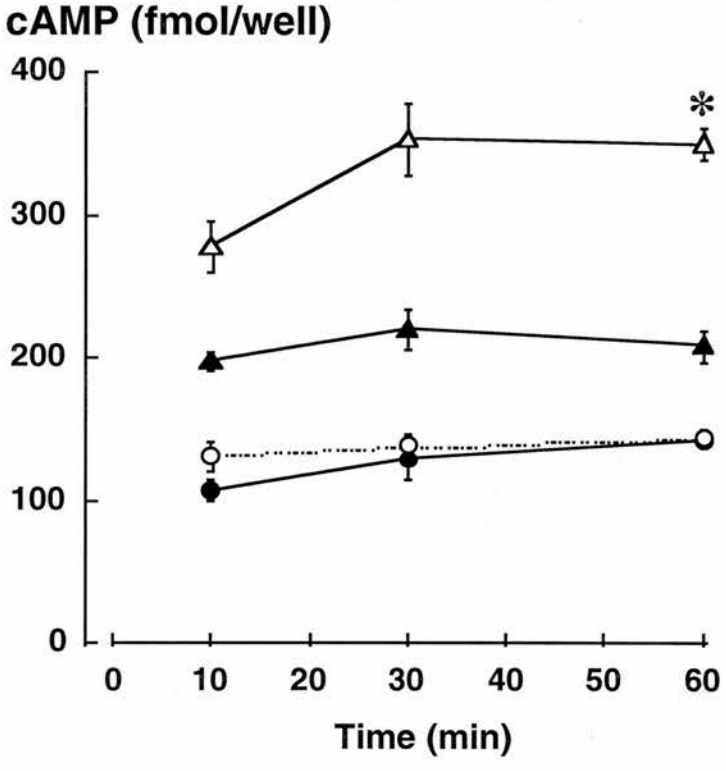

Figure 4.11: Basal cAMP accumulation (*filled circles*); 0.1nM CRF (*filled triangles*), 0.1mM $CdSO_4$ (Cd^{2+}, *empty circles*) and CRF+ Cd^{2+} (*empty triangles*) induced cAMP accumulation over 60 min. Data represent means±SEM, n=3/group. Basal cAMP accumulation at 0 min is 117.46±5.90 fmol/well. *P<0.05 when compared to cAMP response elicited by CRF+ Cd^{2+} at 10 min. Results shown are representative of 2 experiments.

4.1.5c *More specific KvLQT1/IsK and HERG-type K^+-channel inhibitors failed to modify corticosterone inhibition*

As clofilium and astemizole are not highly specific inhibitors of KvLQT1/IsK-type (Folander *et al.*, 1990; Yamagishi *et al.*, 1995) and HERG-type K^+-channel inhibitors (Berul & Morad, 1995), the effects of more specific inhibitors of these K^+-channels were investigated.

Increasing concentrations of chromanol 293B (a specific KvLQT1/IsK-type K^+-channel inhibitor, (Bleich *et al.*, 1997; Busch *et al.*, 1996; Suessbrich *et al.*, 1996)) and WAY-123,398 [a potent inhibitor of the cardiac delayed rectifier K^+-current, (Ellingboe *et al.*, 1992; Spinelli *et al.*, 1993)], did not significantly modulate the ACTH response elicited by 0.1nM CRF in the absence and presence of 100nM corticosterone pretreatment (Figure 4.12). Qualitatively similar results were obtained with the specific HERG-type K^+-channel inhibitors, dofetilide (Carmeliet, 1992; Duff *et al.*, 1995; Fiset *et al.*, 1996) and E4031 (Feng *et al.*, 1997; Heath & Terrar, 1996; Sanguinetti & Jurkiewicz, 1990) (Figure 4.13). The effects of these 4 inhibitors on corticosterone (100nM) inhibition of 0.1nM CRF-stimulated ACTH release are summarized in Table 4.6 (chromanol 293B and WAY-123398) and Table 4.7 (dofetilide and E4031). In contrast to 10μM clofilium, 50μM chromanol 293B and 10μM WAY-123,398 had no significant effect on 100nM corticosterone inhibition of the 0.1nM CRF-elicited ACTH response (Table 4.6). The HERG-type K^+-channel blockers dofetilide (10μM) and E4031 (10μM) similarly had no

discernible effect on 100nM corticosterone inhibition, in contrast to 10μM astemizole (Table 4.7).

The data shown here appears to exclude the specific involvement of KvLQT1/IsK-type and HERG-type K^+-channels in corticosteroid inhibition of stimulated ACTH response in normal rat corticotrophs.

Figure 4.12

Effect of anti-IsK-type and delayed rectifier K^+-channel blockers on CRF-induced ACTH response in cultured rat anterior pituitary cells

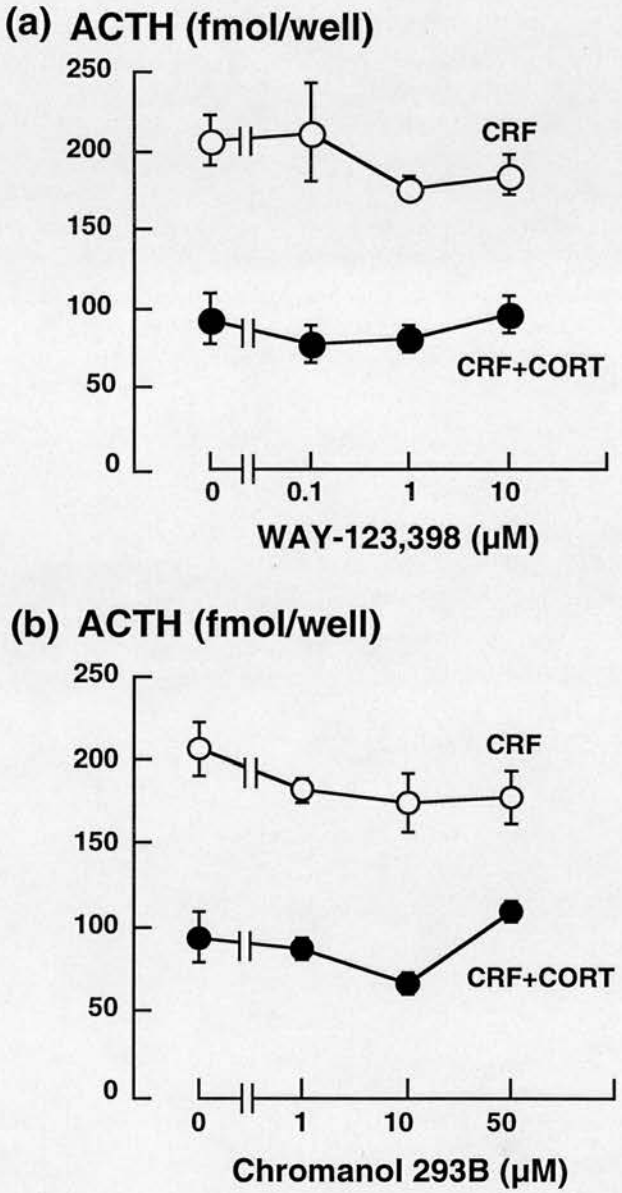

Figure 4.12: Effect of (a) WAY-123,398 or (b) chromanol 293B on 0.1nM CRF-evoked ACTH response in the absence and presence of 100nM corticosterone (CORT) pretreatment. Data represent means±SEM, n=4-6/group. Basal ACTH release is 72.41±11.06 fmol/well. ACTH release elicited by 10μM WAY 123,398 or 50μM chromanol 293B alone are 66.51±11.59 fmol/well and 82.05±23.10 fmol/well respectively. Where error bars are not visible, the SEM bar is less than the space required for the symbol. Results shown are representative of 2 experiments.

Figure 4.13

Effect of anti-HERG-type K^+-channel blockers on CRF-induced ACTH response in cultured rat anterior pituitary cells

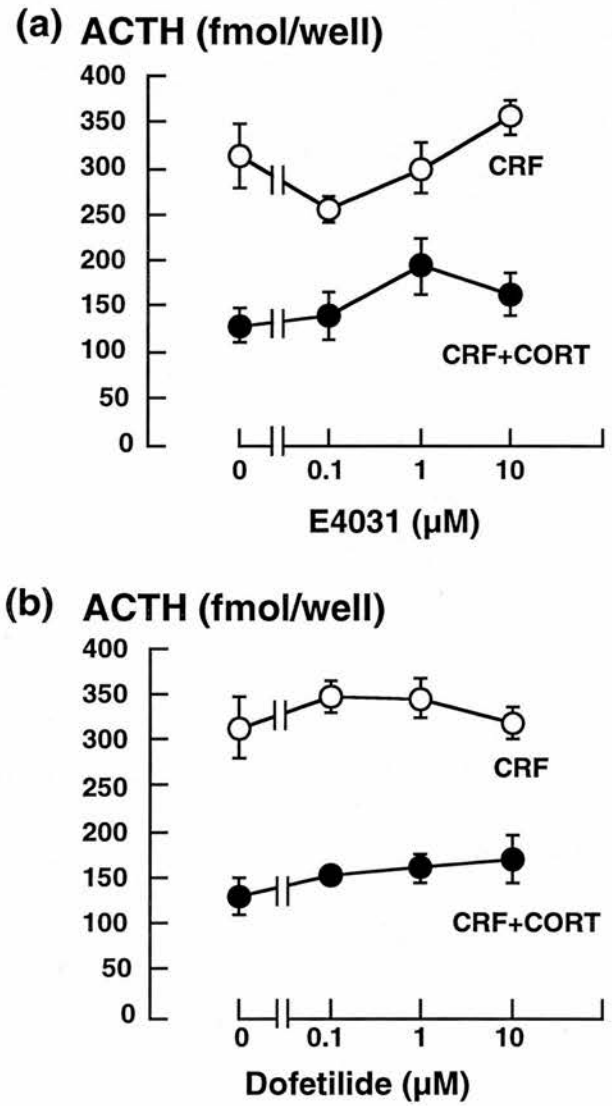

Figure 4.13: Effect of (a) E4031 or (b) dofetilide on ACTH response to 0.1nM CRF in the absence and presence of 100nM corticosterone (CORT) pretreatment. Data represent means±SEM, n=4/group. Basal ACTH release is 51.28±4.87 fmol/well. ACTH release elicited by 10μM E4031 or 10μM dofetilide alone are 55.69±8.43 fmol/well and 53.98±5.96 fmol/well respectively. Results shown are representative of 2 experiments.

Table 4.6

Specific cardiac delayed rectifier (KvLQT1/IsK-type) K^+-channel inhibitors did not alter corticosterone inhibition of CRF-stimulated ACTH release in normal rat corticotrophs

	Veh	293B	WAY	CLOF
CRF	206.86 ± 15.37 *(100 ± 11.43 %)*	178.66 ± 16.12 *(100 ± 15.17 %)*	184.97 ± 13.35 *(100 ± 11.86 %)*	256.79 ± 4.13 *(100 ± 2.24 %)*
CRF+CORT	93.77 ± 15.36 *(15.89 ± 11.42 %)*	109.30 ± 6.08 *(34.72 ± 5.73 %)*	96.80 ± 11.47 *(21.67 ± 10.19 %)*	236.29 ± 12.57 *(88.88 ± 6.82 %)* *

Table 4.6: Effect of 50μM chromanol 293B (293B), 10μM WAY-123398 (WAY) and 10μM clofilium (CLOF) on 100nM corticosterone (CORT) inhibition of 0.1nM CRF-stimulated ACTH release. Data show ACTH release expressed in fmol/well and represent means±SEM (n=5-6/group). Basal release was 72.41±11.06 fmol/well. Values in parentheses represent percentages of control ACTH release (means±SEM, n=4/group), as defined in section 2.7.2. Statistical analysis of the effect of CORT was carried out on the data expressed as percentages (values in parentheses) of the respective stimulus-evoked release. *$p<0.05$ when compared to CRF+CORT alone, 1-way-ANOVA followed by Student-Newman-Keuls' test. Results shown are representative of 2 experiments.

Table 4.7

Inhibition of HERG-type K^+- channels did not alter corticosterone inhibition of CRF-stimulated ACTH release in normal rat corticotrophs

	Veh	E4031	Dofetilide	AST
CRF	183.39 ± 5.42 *(100 ± 4.08 %)*	200.95 ± 6.48 *(100 ± 4.30 %)*	213.20 ± 16.98 *(100 ± 10.43 %)*	236.29 ± 15.71 *(100 ± 8.45 %)*
CRF+CORT	89.37 ± 6.44 *(29.30 ± 4.84 %)*	110.29 ± 14.62 *(39.78 ± 9.71 %)*	108.94 ± 12.72 *(35.96 ± 7.82 %)*	186.82 ± 17.90 *(73.39 ± 9.63 %)* *

Table 4.7: Effect of 10μM E4031, 10μM dofetilide and 10μM astemizole (AST) on 100nM corticosterone (CORT) inhibition of 0.1nM CRF-stimulated ACTH release. Data show ACTH release expressed in fmol/well and represent means ± SEM (n=4/group). Basal relase was 50.39±3.81 fmol/well. Values in parentheses represent percentages of control ACTH release (means±SEM, n=4/group), as defined in section 2.7.2. Statistical analysis of the effect of CORT was carried out on the data expressed as percentages (values in parentheses) of the respective stimulus-evoked release. *$p<0.05$ when compared to CRF+CORT alone, 1-way-ANOVA followed by Student-Newman-Keuls' test.

4.1.6 *Discussion*

4.1.6a *Are the novel K^+-channels underlying the cardiac delayed rectifier currents of any functional importance in early glucocorticoid feedback inhibition in normal rat corticotrophs?*

Consistent data from pharmacological studies in cultured rat anterior pituitary cells with clofilium and astemizole pointed to the potential involvement of the K^+-channels underlying the cardiac delayed rectifier currents (see section 4.1.5a) in glucocorticoid inhibition in normal rat corticotrophs. Subsequent analysis with WAY-123,398, chromanol 293B, E4031 and dofetilide did not consistently reproduce the effects seen with clofilium and astemizole in these rat anterior pituitary primary cultures.

4.1.6b *KvLQT1/IsK-type K^+-channels*

Chromanol 293B had no effect on the ACTH secretory response to CRF with or without corticosterone pretreatment (Table 4.6). Since this chromanol derivative has been reported to be specific for blocking KVLQT1/IsK-type K^+-channels (Busch *et al.*, 1996; Suessbrich *et al.*, 1996), it appears that these K^+-channels are unlikely to have any functional importance in early glucocorticoid inhibition of the ACTH response elicited by CRF in normal rat corticotrophs. Chromanol 293B is purportedly an open channel blocker (Busch *et al.*, 1997) and the KvLQT1/IsK K^+ currents have a very slow rate of activation (Boyle *et al.*, 1987). However, the apparent lack of effect of chromanol 293B is unlikely to be due to an absence of activated (open) KvLQT1/IsK K^+-channels since the blocker was pretreated for 30

min before fresh drug was added again with secretagogue for a further 1h (see section 2.3 and Figure 2.1). There is also indication that the drug may penetrate the cell to block the K^+-channel (Loussouarn *et al.*, 1997).

With respect to clofilium, initial data (section 4.1.5a) suggested the involvement of KvLQT1/IsK-type K^+-channels in the inhibitory action of glucocorticoid. However, these compounds may modulate other K^+-channels (Berul & Morad, 1995; Folander *et al.*, 1990; Yamagishi *et al.*, 1995) and may even have effects unrelated to K^+-channel blockade (Rabinowitz *et al.*, 1997; Silver *et al.*, 1989). For instance, clofilium has been reported to modulate Ca^{2+}/calmodulin-activated PDE activity through antagonism of calmodulin (Silver *et al.*, 1989). This may explain the observation in preliminary experiments that clofilium had a tendency to decrease CRF-induced cAMP response (Lim, not shown). In addition, a recent study (Rabinowitz *et al.*, 1997) reported the observation that clofilium triggers a sustained increase in acid release at concentrations similar to those used in this study in some human and murine cell lines. Thus, clofilium may produce undesirable effects on cellular metabolism and may account for the cytotoxic effect seen in the cultured rat anterior pituitary cells when treated with higher concentrations (more than 10μM) of clofilium (section 4.1.5a). Furthermore, since Cd^{2+} could not completely block ACTH secretion elicited by the combination of CRF and clofilium (while CRF-induced secretion was completely inhibited, see section 4.1.5b), it is possible that clofilium may perhaps have some non-specific toxicity that caused ACTH leakage from the cultured rat corticotrophs. Nonetheless, it appears that CRF stimulated ACTH release in the presence of clofilium (10μM) treatment (section 4.1.5b) is

largely dependent on Ca^{2+}, suggesting that the ACTH secretory pathway activated by CRF is fundamentally intact. Since clofilium has been known to block other types of K^+-channels besides KvLQT1/IsK-type channels (Folander *et al.*, 1990; Yamagishi *et al.*, 1995), it is likely that the antagonism of glucocorticoid inhibition by clofilium seen in cultured rat anterior pituitary cells may involve modulation of other class(es) of K^+-channels. Moreover, the IsK protein has not been so far found to be expressed in rat brain (Sugimoto *et al.*, 1990). There appears to be only one report to date documenting the possible presence of the IsK protein in rat neurohypophysial nerve terminals based on the observation of KvLQT1/IsK-like K^+ currents (Kilic *et al.*, 1996).

Taken together, these observations suggests that functional KvLQT1/IsK-like K^+-channels are not fundamentally important for glucocorticoid inhibition in normal rat corticotrophs.

4.1.6c *HERG-type K^+-channels*

The methanesulphonanilides E4031 and dofetilide are specific inhibitors of HERG-type K^+-channels (see section 4.1.5c). Both drugs have been found to bind to some form of endogenous receptor (methanesulphonanilide receptor) that is closely associated with HERG-channels (Duff *et al.*, 1995; Sanguinetti *et al.*, 1995). Furthermore, Sanguinetti and colleagues have found that concentrations of E4031 similar to that used in this study failed to block a HERG current expressed in Xenopus oocyte (Sanguinetti *et al.*, 1995). Therefore, it is possible that the apparent lack of modulation of glucocorticoid inhibition in normal rat corticotrophs by E4031

and dofetilide may be due to the absence of an additional subunit required for drug sensitivity.

The second generation H1 antagonist astemizole has been extensively shown to block HERG-type K^+-currents (Suessbrich *et al.*, 1996; Taglialatela *et al.*, 1998; Woosley, 1996), although it has been reported to block A-type transient outward K^+ currents as well (Berul & Morad, 1995). The effects of astemizole on glucocorticoid inhibition of CRF-elicited ACTH response seen in cultured rat anterior pituitary cells (section 4.1.5a) is intriguing given the lack of such an effect by E4031 and dofetilide (section 4.1.5c). It may be possible that some as yet unknown effect related to the antihistaminergic actions of astemizole, is somehow causing the reduction of glucocorticoid inhibition seen in normal rat corticotrophs.

4.1.6d *Conclusion ------ involvement of novel K^+-channels in early glucocorticoid feedback inhibition*

Data presented in this section has shown that clofilium and astemizole consistently and reproducibly attenuated early glucocorticoid inhibition of CRF-stimulated ACTH secretion in primary cultures of rat anterior pituitary cells. If the effects of clofilium and astemizole are indeed through inhibition of K^+-channels, then these K^+-channels are likely to be of novel and as yet unidentified type(s). Furthermore, it is possible that clofilium and astemizole block multiple K^+-channels, including TEA-sensitive and TEA-insensitive K^+-channels. The blockage of both TEA-sensitive and TEA-insensitive K^+-channels may be required to achieve sufficient depolarization that is needed to oppose the effects of glucocorticoid induced proteins in primary

cultures of rat anterior pituitary cells. Hence, when only specific K^+-channel blockers were used (see section 4.1.5c), there was no significant effect on glucocorticoid action. In contrast, blockade of TEA-sensitive K^+-channels was adequate to counteract the inhibitory action of glucocorticoids on CRF-evoked ACTH secretion in AtT20 cells (see section 4.1.2b).

4.2 Analysis of the effect of sustaining cAMP accumulation on early glucocorticoid inhibition in primary cultures of rat anterior pituitary corticotrophs

4.2.1 *Introduction*

Previous studies have demonstrated the importance of the regulation of intracellular cAMP metabolism through Ca^{2+}-dependent feedback in early glucocorticoid inhibition in AtT20 cells (see Section 1.2.4). As this Ca^{2+}-dependent feedback mechanism may also evident in normal rat corticotrophs (Antoni, 1995), the objective of this section of the thesis is to examine the role this Ca^{2+}-mediated feedback mechanism in glucocorticoid inhibition in cultured rat adenophypophysial corticotrophs.

The physiological hypothalamic regulator of ACTH secretion, AVP, has been previously proposed to reduce glucocorticoid feedback *in vitro* (Bilezikjian *et al.*, 1987) and *in vivo* (Antoni, 1993; Deuster *et al.*, 1998; Scaccianoce *et al.*, 1991; Scott & Dinan, 1998; von Bardeleben *et al.*, 1985). However, the underlying cellular mechanism for the resistance of AVP to glucocorticoid action at the pituitary corticotroph are not well understood. Recent studies *cf* (Antoni, 2000) have suggested that in the presence of AVP, the elevation in CRF-mediated cAMP response may be a consequence of bypassing the Ca^{2+}-dependent feedback. Hence, the effects of AVP on CRF-induced cAMP and ACTH responses as well as on glucocorticoid feedback inhibition were analyzed.

Other notable components of the Ca^{2+}-mediated feedback mechanism include PDEs and calcineurin (Ca^{2+}/calmodulin activated protein phosphatase, PP2B). Inhibition of these components could potentially modulate glucocorticoid inhibition of stimulated ACTH release (see section 1.2.4). Therefore, the effects of rolipram (a relatively selective inhibitor of cAMP-dependent PDE) and immunosuppressant drugs that inhibit calcineurin, were tested.

Results

4.2.2 Sustaining CRF-induced cAMP at elevated levels with AVP

4.2.2a *cAMP response to CRF and AVP*

There was no effect of 2nM AVP on intracellular cAMP levels at any time point tested (Figure 4.14a). The cAMP levels increased 2.5-fold over basal in response to 0.1nM CRF and remained unchanged over 60 min. AVP enhanced CRF-induced cAMP accumulation at all time points examined, and although cAMP levels measured at 60 min were lower when compared with levels at 10 min, they were still 3 times higher than with CRF alone ($p<0.05$, n=4/group, 1-way-ANOVA, Dunnett's test) (Figure 4.14a).

In summary, the data show the significant enhancement of CRF-induced cAMP accumulation in the presence of AVP over 60min.

4.2.2b *CRF-evoked ACTH response was resistant to corticosterone inhibition in the presence of AVP*

AVP (2nM) alone had no significant effect on basal ACTH release but markedly potentiated the ACTH response to 0.1nM CRF (Figure 4.15a). Corticosterone (10-100nM) dose-dependently inhibited 0.1nM CRF-evoked ACTH release (Figure 4.15b). The degree of corticosterone (10-100nM) inhibition of ACTH release elicited by a combination of 0.1nM CRF and 2nM AVP was markedly attenuated (Figure 4.15b).

Hence, the synergistic cAMP response elicited by CRF and AVP correlates with the potentiation in ACTH release that is associated with a marked resistance to corticosterone inhibition.

4.2.3 Sustaining CRF-induced cAMP at elevated levels with rolipram

4.2.3a *cAMP response to CRF and rolipram*

The type 4 PDE inhibitor rolipram (0.1mM) elicited a small but significant increase (1.7-fold) in cAMP accumulation over basal cAMP levels. Basal cAMP accumulation remained unchanged while the cAMP response to 0.1mM rolipram significantly increased over 60 min (Figure 4.14b). CRF (0.1nM)-induced cAMP accumulation remained unchanged and was consistently potentiated by 0.1mM rolipram over 60 min. The cAMP response elicited by a combination of 0.1nM CRF and 0.1mM rolipram significantly increased over 60 min (Figure 4.14b).

4.2.3b *Corticosterone inhibition of CRF-induced ACTH release was reduced by rolipram*

CRF (0.1nM) elicited a 2.5-fold increase over basal ACTH release while 0.1mM rolipram had no significant effect on basal ACTH release (Figure 4.16a). Similar to the effect of 2nM AVP, 0.1mM rolipram produced a synergistic increase of 0.1nM CRF-evoked ACTH release (Figure 4.16a). The combination of 0.1nM CRF and 0.1mM rolipram elicited an ACTH response that was much less effectively inhibited by corticosterone (10-100nM) (Figure 4.16b).

These results show that rolipram potentiated the cAMP response elicited by CRF. This correlates with the potentiation in ACTH release and is associated with a marked resistance to corticosterone inhibition.

Figure 4.14

Potentiation of CRF-induced cAMP accumulation by (a) AVP and (b) rolipram in cultured rat anterior pituitary cells

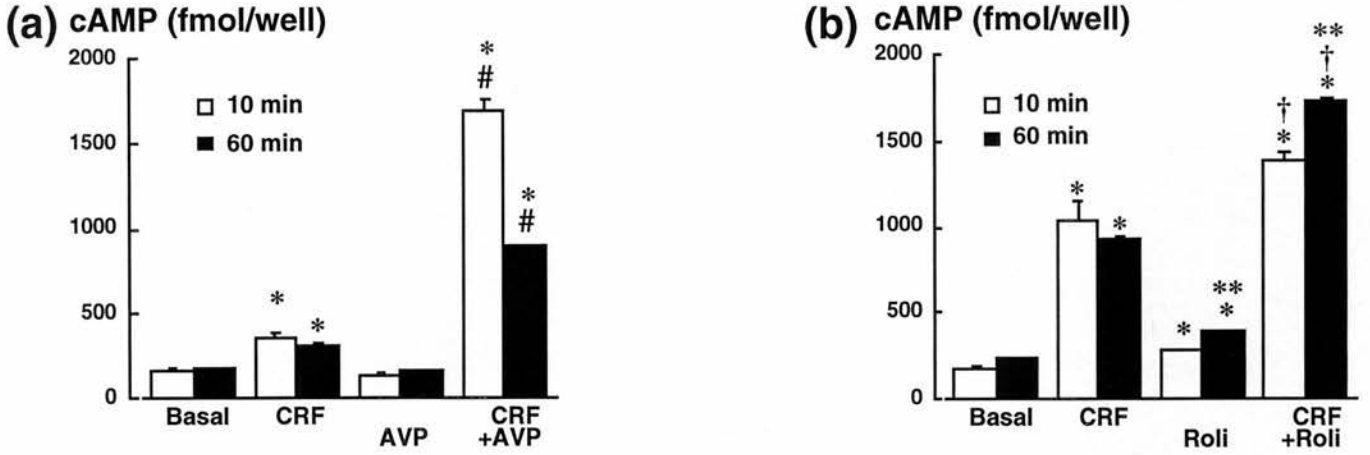

Figure 4.14: Measurement of cAMP accumulation in cultured rat anterior pituitary cells. Columns are means, bars indicate SEM, n=4/group. *P<0.05 when compared to basal cAMP levels measured at corresponding time points, 1-way ANOVA followed by Newman-Keuls test. (a) Basal cAMP level measured at 0 time point is 153.11±7.44 fmol/well. #P<0.05 for the interaction between 0.1nMCRF and 2nM AVP, 2-way ANOVA. (b) Basal cAMP level measured at 0 time point is 205.72±10.18 fmol/well. †P<0.05 for the interaction between 0.1nM CRF and 0.1mM rolipram (Roli). **P<0.05 when compared to corresponding cAMP accumulation measured at 10min. Results shown are representative of 2 experiments.

Figure 4.15

Antagonism of corticosterone inhibition of CRF-evoked ACTH response by AVP in primary cultures of rat anterior pituitary cells

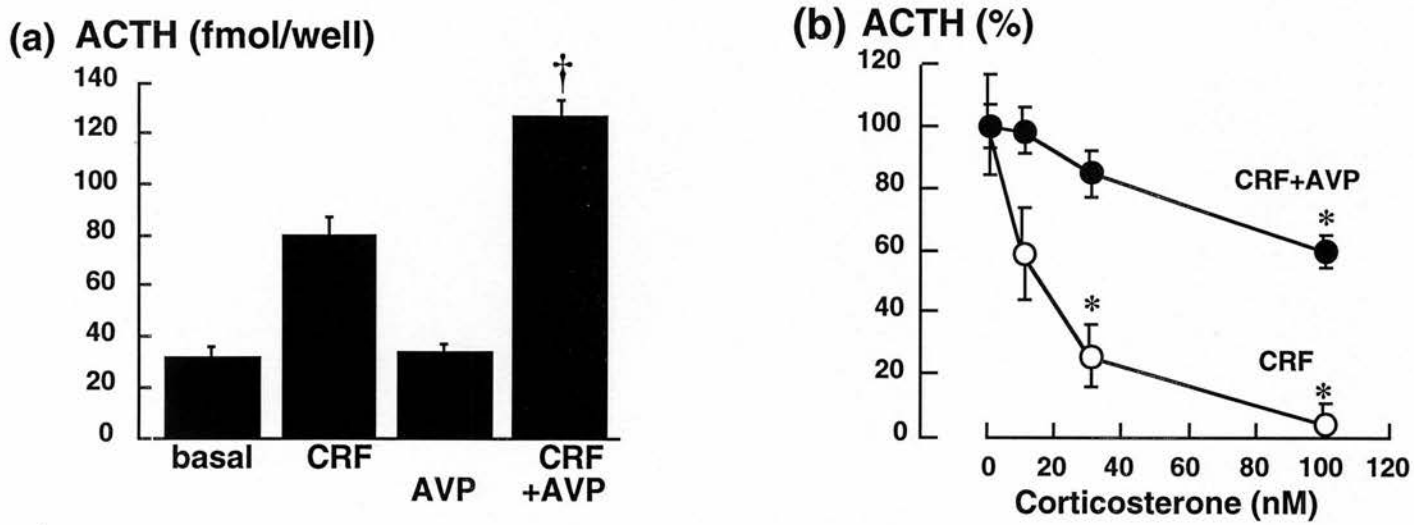

Figure 4.15: (a) ACTH response elicited by 0.1nM CRF and 2nM AVP. Solid columns are means, bars indicate SEM, n=4/group. †P<0.05 for the interaction between CRF and AVP, 2-way ANOVA. (b) Concentration dependent inhibition of 0.1nM CRF-evoked ACTH release by corticosterone in the absence and presence of 2nM AVP. Data are expressed as percent of control ACTH release (defined in section 2.3.1) and represent means±SEM, n=4/group. *P<0.05 when compared to corresponding control group (i.e. without corticosterone pretreatment), 1-way ANOVA followed by Dunnett's test. Results shown are representative of 8 experiments.

Figure 4.16

Rolipram altered corticosterone inhibition of CRF-induced ACTH release by cultured rat anterior pituitary cells

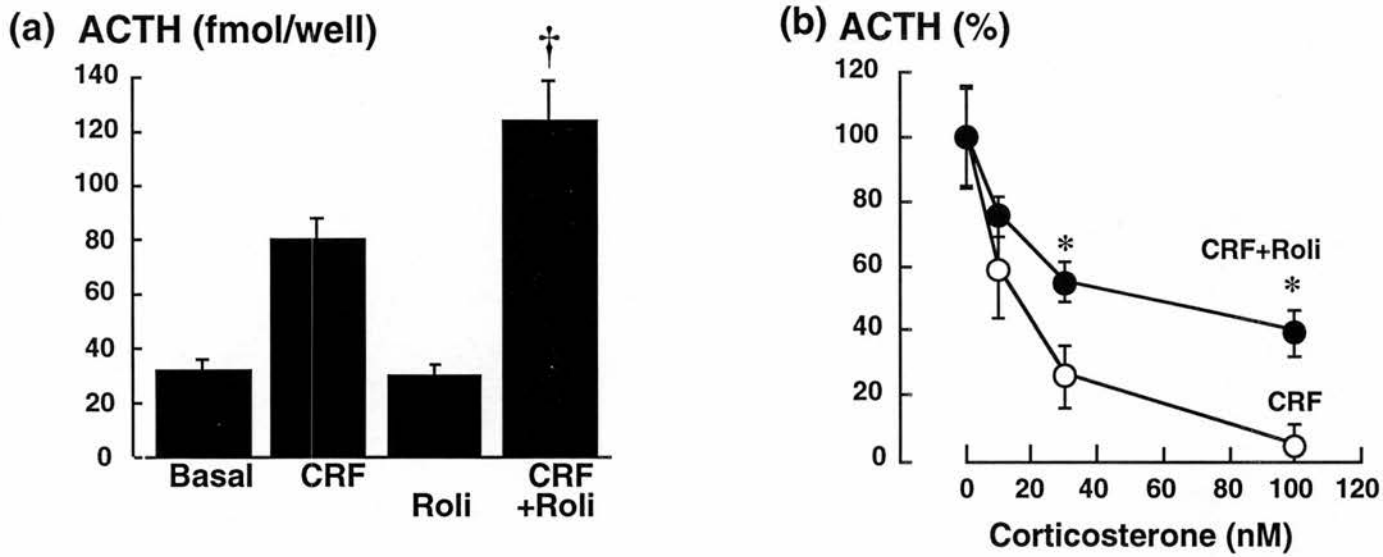

Figure 4.16: (a) ACTH response to 0.1nM CRF and 0.1mM rolipram (Roli). Solid columns are means, bars indicate SEM, n=4/group. †P<0.05 for the interaction between CRF and rolipram, 2-way ANOVA. (b) Concentration dependent inhibition of 0.1nM CRF-evoked ACTH release by corticosterone in the absence and presence of 0.1mM rolipram. Data are expressed as percent of control ACTH release (defined in section 2.3.1) and represent means±SEM, n=4/group. *P<0.05 when compared to corresponding CRF group, 1-way ANOVA followed by contrast of means. Results shown are representative of 4 experiments.

4.2.4 *Discussion*

The data presented in this section demonstrate that exaggerated cAMP responses lead to resistance to glucocorticoid inhibition of CRF-stimulated ACTH secretion. This effect of blunting the glucocorticoid response could be achieved by using either AVP or rolipram.

Previous studies have presented a disparate picture of the effect of glucocorticoids on secretagogue-induced cAMP accumulation in the anterior pituitary corticotroph. Several studies have found that glucocorticoids may decrease CRF-stimulated cAMP production by up to 50% (Bilezikjian & Vale, 1983; Sobel, 1985; Vale *et al.*, 1983). However, cells were exposed to glucocorticoids for much longer than 2 h (the duration of glucocorticoid exposure in our studies) in these studies. In contrast, other studies have shown that glucocorticoids have no effect on CRF-evoked cAMP accumulation (Abou-Samra *et al.*, 1986; Dartois & Bouton, 1986; Giguère *et al.*, 1982), suggesting that the steroid acts at a step subsequent to cAMP production and accumulation.

The synergistic ACTH response to CRF and AVP is well established *in vitro* and in *vivo* (Brooks & Challis, 1989; DeBold *et al.*, 1984; Keller-Wood, 1998; Milsom *et al.*, 1985). In anterior pituitary corticotrophs (Koch & Lutz-Bucher, 1989; Murakami *et al.*, 1984; Vale *et al.*, 1983), the potentiation of CRF-induced cAMP and ACTH by AVP appears to be a robust and physiologically relevant phenomenon (Rivier &

Vale, 1983). This is despite the fact that different concentrations of CRF and AVP, as well as different durations of stimulation by these secretagogues used in various studies (Abou-Samra *et al.*, 1987; Bilezikjian *et al.*, 1987; Carvallo & Aguilera, 1989; Hoffman *et al.*, 1985; Yates *et al.*, 1971). In our experiments, we used physiological concentrations of AVP (2nM) and CRF (0.1nM) to demonstrate the synergistic stimulation of cAMP accumulation and ACTH secretion elicited by CRF in primary cultures of anterior pituitary corticotrophs (see section 4.2.2a). When cells were stimulated with CRF and AVP concomitantly, the inhibitory effect of corticosterone on stimulated ACTH secretion was markedly attenuated (section 4.2.2b). The role of AVP in modulating glucocorticoid feedback and subsequent alteration of the HPA axis activity may have important implications for understanding the pathophysiology of diseases like depression (Scott & Dinan, 1998). Thus, it would be important to understand the mechanism by which AVP potentiates CRF-induced ACTH secretion, and which is highly resistant to glucocorticoid inhibition. It has been shown that protein kinase C (PKC) plays a principal role in mediating the potentiating effects of AVP on CRF-stimulated ACTH secretion and cAMP accumulation at physiological levels in corticotrophs (Abou-Samra *et al.*, 1986; Abou-Samra *et al.*, 1987; Carvallo & Aguilera, 1989; King & Baertschi, 1990). Work in anterior pituitary homogenates has shown that AVP reduces PDE activity by 30%, plausibly through PKC and may partly explain the experimental observation that AVP potentiates CRF-induced cAMP accumulation (Abou-Samra *et al.*, 1987).

Whether early glucocorticoid inhibition of stimulated ACTH secretion targets cAMP accumulation/production in the corticotroph remains debatable. Data presented here show that ACTH secretion elicited by non-hydrolyzable cAMP analogues like CPT-cAMP is inhibited by glucocorticoids (section 4.1.2a and 4.1.2c), consistent with data from other studies (Bilezikjian & Vale, 1983; Giguère *et al.*, 1982). Therefore, it appears that glucocorticoid inhibition may not involve modulation of cAMP levels per se. Instead, the intracellular mediators of glucocorticoid inhibition could include enzymes involved in the metabolism of cAMP.

Our data with rolipram (section 4.2.3) suggest that the type IV cAMP-dependent PDE is present in normal rat corticotrophs. By blunting the downregulation of cAMP signals (through inhibition with rolipram) by PDE IV, intracellular cAMP was elevated to levels that could reduce early glucocorticoid inhibition.

Therefore, using different physiological means (AVP and rolipram) to induce higher than usual levels of cAMP in cultured rat anterior pituitary cells, early glucocorticoid inhibition was significantly attenuated. cAMP is the primary mediator of CRF-induced ACTH secretory pathway in corticotrophs. Its main effector, PKA modulates distinct components in the pathway to increase $[Ca^{2+}]_I$ which ultimately leads to ACTH secretion (see section 1.2.4). It appears that driving cAMP to exaggerated levels (by the physiological methods mentioned above) can potentially overcome the inhibitory effect of glucocorticoids on components of the secretory pathway. For instance, cAMP acting through PKA may inhibit K^+-channels and

cause cell depolarization. This is in opposition to the effect of glucocorticoids, which appears to be maintenance of the membrane potential.

4.3 Analysis of the role of protein phosphorylation in early glucocorticoid inhibition of ACTH release

4.3.1 *Introduction*

Previous pharmacological evidence have strongly indicated that calcineurin, a Ca^{2+}/calmodulin activated serine/threonine phosphatase (protein phosphatase 2B) may be involved in glucocorticoid early inhibition in AtT20 cells [see section 1.3.3 and (Shipston *et al.*, 1994)]. Hence, immunosuppressants such as FK506 and cyclosporin A (CsA), potent inhibitors of calcineurin (Liu *et al.*, 1991; Schreiber, 1992), were tested to examine whether these drugs also modulate glucocorticoid inhibition. in cultured rat anterior pituitary cells. FK506 and CsA act by binding to soluble intracellular receptor proteins, which are the FK506 binding protein-12 (FKBP-12) and cyclophilin respectively and inhibits their *cis-trans* peptidyl-prolyl isomerase (PPIase) activity . These ligand-immunophilin complexes binds and inhibits the phosphatase activity of calcineurin (Kunz & Hall, 1993; Liu *et al.*, 1991; Schreiber, 1992) (independently of their effect on PPIase, (Schreiber, 1991; Sigal & Dumont, 1992), by possibly excluding phosphopeptide binding in the enzyme's active site (Clipstone *et al.*, 1994; Salowe & Hermes, 1998). The compound, L-685,818, which binds and inhibits the PPIase activity of FKBP-12 but has no effect on calcineurin, was tested as a control to show that the effects of FK506 are elicited through inhibition of calcineurin.

Results

4.3.2 Effects of immunosuppressants on stimulated ACTH secretion

4.3.2a *ACTH response to increasing concentrations of FK506*

Analysis of increasing concentrations of FK506 showed that 2μM FK506 alone elicited a small (1.6-fold) but statistically significant increase over basal ACTH release and caused a significant enhancement of the ACTH response to 0.3nM CRF (Figure 4.17a). No effect of 0.08μM and 0.4μM FK506 on 0.3nM CRF-stimulated ACTH release was observed (Figure 4.17a).

4.3.2b *Potency of corticosterone inhibition was markedly reduced by immunosuppressant drugs*

CRF (0.1nM) elicited an ACTH response that was dose-dependently inhibited by corticosterone (10-100nM) (Figure 4.17b). Pretreatment with 2μM FK506 reduced the apparent potency of corticosterone (10-100nM) to inhibit 0.1nM CRF-evoked ACTH response by more that 10-fold (Figure 4.17b).

FK506 (2μM) and cyclosporin A (2μM) had no significant effect on basal ACTH release. Both drugs potentiated the ACTH response to 0.1nM CRF and was significantly less inhibited by 100nM corticosterone (Table 4.8). The compound L-685,818 had no significant effect on basal and 0.1nM CRF-induced ACTH release

and also did not modify the corticosterone (100nM) inhibition of 0.1nM CRF-evoked ACTH response (Table 4.8).

To summarize, the data show that inhibition of calcineurin markedly attenuates early glucocorticoid inhibition of stimulated ACTH release in normal rat corticotrophs, extending the observations made in AtT20 cells.

Figure 4.17

CRF-induced ACTH response was less effectively inhibited by corticosterone in the presence of FK506 in cultured rat anterior pituitary cells

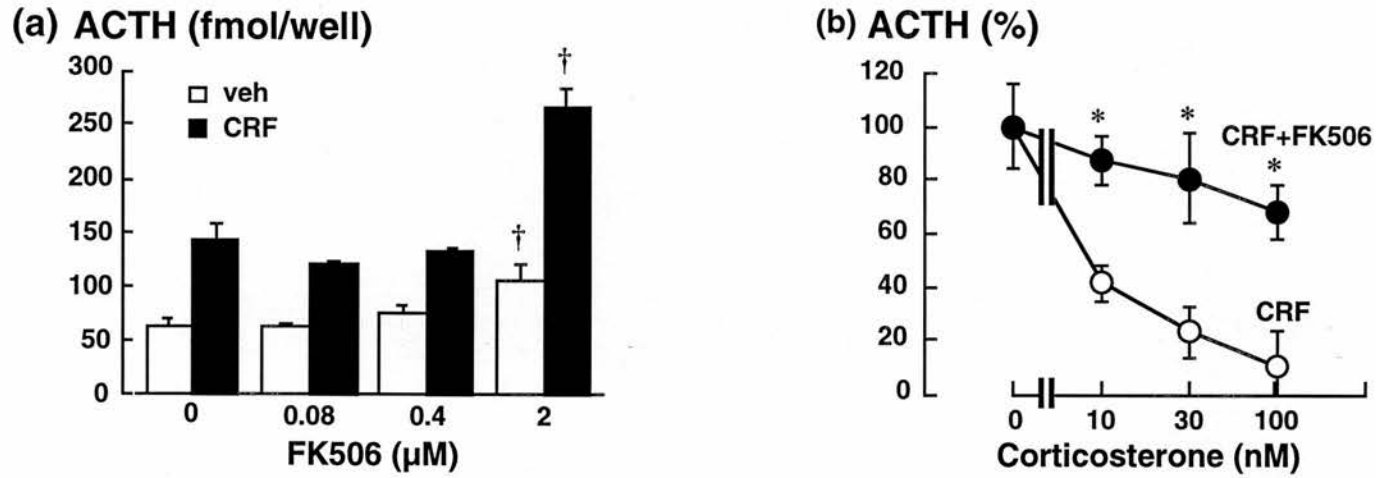

Figure 4.17: (a) Concentration dependent stimulation of ACTH release by FK506 in vehicle and CRF treated cells. Columns are means, bars indicate SEM, n=4/group. †P<0.05 when compared to corresponding control group (without FK506), 1-way ANOVA followed by Dunnett's test. (b) Concentration dependent inhibition of 0.1nM CRF-evoked ACTH release by corticosterone in the absence and presence of 2μM FK506. Data are expressed as percent of control ACTH release (as defined in section 2.3.1) and are means±SEM, n=4/group. *P<0.05 when compared to corresponding CRF group, 1-way ANOVA followed by contrast of means. FK506 was added during the last 30 min of corticosterone pretreatment. Results shown are representative of 2 experiments.

Table 4.8

Inhibition of CRF-stimulated ACTH release by corticosterone in normal rat corticotrophs was reduced by immunosuppressant drugs

	Vehicle	+ CRF	+ CRF/CORT	% inhibition
Vehicle	47.13 ± 2.87	97.53 ± 6.67	55.48 ± 3.77	83.43 ± 7.49
FK506	55.61 ± 4.36	163.61 ± 17.64 †	108.01 ± 5.50	47.74 ± 4.72 *
CsA	56.30 ± 7.82	172.60 ± 21.55 †	113.40 ± 15.06	47.19 ± 12.01 *
L-685,818	62.88 ± 8.41	97.20 ± 7.73	50.50 ± 1.09	93.27 ± 2.18

Table 4.8: Effect 100nM corticosterone (CORT) on 0.1nM CRF-stimulated ACTH release in the presence of 2μM FK506, 2μM cyclosporin A (CsA) and 20μM L-685,818 in rat anterior pituitary cells cultured in serum-free medium (defined in section 2.2) supplemented with 4nM CORT. FK506, CsA and L-685,818 were added during the last 30 min of CORT pretreatment. Data show ACTH release expressed in fmol per well or % inhibition, which represents the percentage inhibition of control ACTH release by CORT as defined in section 2.7.2. Values represent means±SEM (n=4/group). Statistical analysis of the effect of CORT was carried out on the data expressed as % inhibition. * $p<0.05$ when compared to % inhibition of CRF-stimulated ACTH release, 1-way-ANOVA followed by Dunnett's test. † $p<0.05$ for the interaction between CRF and the respective drugs, 2-way-ANOVA. Results shown are representative of 2 experiments.

4.3.3 *Discussion*

The results presented in this section provide strong pharmacological evidence that calcineurin is an important component of early glucocorticoid inhibition of stimulated ACTH secretion in cultured rat anterior pituitary cells. This is consonant with previous pharmacological evidence obtained in AtT20 cells (Shipston *et al.*, 1994).

4.3.3a *Potential functions of calcineurin in pituitary cells*

One of the first physiologically relevant functions implicated for calcineurin in pituitary corticotrophs was an inhibitory role in ACTH secretion in AtT20 cells. Consistent with previous findings in AtT20 cells (Antoni *et al.*, 1993), FK506 significantly enhanced the ACTH secretory response to CRF in normal rat corticotrophs, suggesting a similar inhibitory role for calcineurin in stimulus-secretion coupling. Greater understanding of the role of calcineurin in other anterior pituitary cell types is emerging. A recent study in cultured rat anterior pituitary cells found that the stimulation of growth hormone release from somatotroph cells by FK506 was mediated through inhibition of calcineurin which resulted in the activation of cAMP-dependent protein kinase signally pathways (Ohye *et al.*, 1998). In rat gonadotrophs, calcineurin has been localized to the cytosolic and nuclear fractions and is postulated to mediate the actions of gonadotrophin release hormone (Natarajan *et al.*, 1991). Calcineurin has also been implicated in the regulation of cell proliferation in cultured pituitary cells (Florio *et al.*, 1996). The regulation of Ca^{2+}-channel activity and exocytosis in rat lactotrophs is reciprocally controlled by protein kinase C and calcineurin (Fomina & Levitan, 1997).

4.3.3b *Potential targets for calcineurin regulation and their relevance to early glucocorticoid inhibition in normal rat corticotrophs*

Adenyl Cyclase 9

Previous work has shown that CRF-stimulated cAMP accumulation in AtT20 cells is inhibited by intracellular Ca^{2+} and that this effect was suppressed by FK506 (Antoni *et al.*, 1995). This suggested that calcineurin formed a Ca^{2+} sensor negative feedback loop on CRF-stimulated cAMP accumulation. Importantly, the inhibition of calcineurin by FK506 (which would result in a disruption of the Ca^{2+}-dependent negative control of cAMP levels) was associated with a marked reduction in the potency of dexamethasone to inhibit CRF-elicited ACTH release (Shipston *et al.*, 1994). Therefore, it appears that the mechanism of glucocorticoid inhibition may involve modulation of this Ca^{2+} feedback control of cAMP through calcineurin in AtT20 cells. Further studies established an association of the effect of calcineurin on cAMP synthesis with the expression of a novel adenylyl cyclase isotype (AC9) (Antoni *et al.*, 1995), identified as the major isotype in AtT20 cells and a potential target of calcineurin-mediated inhibition of cAMP synthesis (Antoni *et al.*, 1998; Paterson *et al.*, 1995). Although the notion that AC9 is modulated by calcineurin is controversial since other workers (Premont *et al.*, 1996) have reported no effect of Ca^{2+}/calmodulin on AC9 over-expressed in Sf9 insect cells, observations on the primary structure of AC9 support the notion that calcineurin regulates AC9 (Antoni *et al.*, 1998).

The Ca^{2+} feedback control of intracellular cAMP is likely to be operative in normal rat corticotrophs as well. Firstly, mRNA for AC9 is also present at relatively high levels in rat adenohypophysis (Smith, SM, Paterson, JM, Simpson, J & Antoni, FA, unpublished). Secondly, in acutely dispersed rat adenohypophysial cells, depletion of rapidly mobilisable Ca^{2+} stores produced a marked enhancement of the cAMP response to CRF (Antoni, 1997; Antoni, 1995). My own studies described in section 4.1.5b showed that Cd^{2+} significantly increased the cAMP accumulation elicited by CRF in cultured rat anterior pituitary cells. As Cd^{2+} blocks Ca^{2+} through most known Ca^{2+}-channels, these findings are in agreement with the notion that Ca^{2+} inhibits CRF-induced cAMP accumulation.

Taking together the data presented in this section and other work done in this laboratory (Antoni *et al.*, 1995; Antoni *et al.*, 1998; Paterson *et al.*, 1995; Shipston *et al.*, 1994), it is reasonable to hypothesize that calcineurin may also target adenylyl cyclase and mediate the Ca^{2+}-dependent control of cAMP metabolism in normal rat corticotrophs. Importantly, glucocorticoid may act through calcineurin (potentially through calmodulin) to sensitize the corticotroph to the Ca^{2+} negative feedback mode, prematurely terminating Ca^{2+} signals to levels insufficient for ACTH release.

Other potential sites for calcineurin regulation

Calcineurin has also been implicated in the control of voltage-gated ion channel activity by modulation of their phosphorylation states (Fomina & Levitan, 1997; White *et al.*, 1991). In normal rat corticotrophs, it is evident that some class of K^{+}-channels (though yet to be identified) are vital to early glucocorticoid inhibition.

These may be substrates for the phosphorylatory control by calcineurin, which is in turn targeted by glucocorticoids.

5

SUMMARY AND PERSPECTIVES

5

SUMMARY AND PERSPECTIVES

5.1 Introduction

The principal purpose of this thesis was to investigate the mechanisms of action by which early (within 2h) glucocorticoid feedback inhibition of stimulated ACTH secretion occurs in normal anterior pituitary corticotrophs. This involved verifying a model for the intracellular mechanism of early glucocorticoid inhibition proposed based on experiments carried out in the murine corticotroph tumour cell line AtT20 in normal rat corticotrophs.

The following sections will summarize the key experimental findings. Detailed discussions are found in the relevant sections of Chapter 3 and 4. Further lines of investigation that can be pursued based on research findings presented in this thesis will then be proposed under corresponding sections.

5.2 Role of calmodulin in early glucocorticoid feedback inhibition

Experiments to investigate whether calmodulin functions as a primary mediator of early glucocorticoid feedback inhibition were carried out in AtT20 cells. Two approaches were used, namely a constitutive calmodulin over-expression system and

an inducible calmodulin over-expression system were generated in AtT20 cells. These transfected AtT20 cells were then analyzed for their calmodulin mRNA levels as well as ACTH secretion characteristics.

Using Northern analysis, several transfected AtT20 clones exhibited elevated levels of calmodulin mRNA. Functional protein analysis also indicated increased calmodulin activity in these clones. However, the ACTH response to CRF in these cells was not significantly different from those of control, wild type AtT20 cells or cells transfected with the vector alone. It was thus not possible to conclude whether calmodulin may mediate the early inhibitory actions of glucocorticoids in corticotrophs. The inducible calmodulin over-expression system set up in AtT20 cells were subsequently found not to be suitable for experimentation since the doxycycline (the compound used for inducing calmodulin expression) inhibits ACTH secretion.

Other mammalian inducible expression systems have since become commercially available. Of note is the ecdysone-inducible expression system from Invitrogen, which drives gene expression with the analogues of an insect steroid hormone ecdysone. As Ponasterone A and Muristerone A (the ecdysone analogues) are reportedly inert to mammalian physiology, the ecdysone-inducible system may be useful for driving the transient over-expression of calmodulin in AtT20 cells and will not interfere with the assessment of any modulation of ACTH secretion in these cells. Therefore, the role of calmodulin as a primary mediator of early glucocorticoid

inhibition could be better explored using other mammalian inducible expression systems such as the above-mentioned one.

5.3 Role of K^+-channels in early glucocorticoid feedback inhibition in normal rat corticotrophs

Experiments in AtT20 cells have suggested that the BK channels are pivotal for early glucocorticoid inhibition of CRF-stimulated ACTH secretion. Hence, the role of BK-channels in glucocorticoid inhibition was investigated in normal rat corticotrophs.

Pharmacological analysis of K^+-channels in primary cultures of rat anterior pituitary cells showed that blocking BK-channels with charybdotoxin did not significantly alter the efficiency of early glucocorticoid inhibition of CRF-stimulated ACTH secretion. Thus, BK-channels do not appear to have an important role in glucocorticoid inhibition in normal rat corticotrophs, in contrast to AtT20 cells. Therefore, further pharmacological analysis was carried out using blockers of other types of K^+-channels, namely clofilium and astemizole. In the presence of these K^+-channels inhibitors, glucocorticoid inhibition of CRF-stimulated ACTH release was significantly blunted in cultured rat anterior pituitary cells. This suggested that there is probably a population of ion channels (K^+-channels) important for maintaining the membrane potential. In particular, the HERG-type and KvLQT1/IsK-type K^+-channels may be involved. More specific inhibitors for these K^+-channels were

subsequently tested in cultures of rat anterior pituitary cells. These specific inhibitors did not modulate the inhibitory efficacy of glucocorticoid.

As clofilium and astemizole are relatively unspecific blockers of K^+-channels, further studies are needed to clarify whether K^+-channels are indeed important for early glucocorticoid feedback inhibition using a combination of molecular biological, biochemical and electrophysiological methods. For instance, a single corticotroph pre-treated with glucocorticoids can be used for patch-clamp studies to investigate if K^+-currents are modulated by steroid treatment. Additionally, the availability of pharmacological blockers of K^+-channels with greater specificities will also help to shed light on the identities of the K^+-channels important for early glucocorticoid inhibition in normal rat corticotrophs.

A further aspect is the involvement of K^+-channels in early glucocorticoid action concerns protein phosphorylation. In particular, glucocorticoid-induced proteins may directly or recruit other signaling pathways to regulate K^+-channel activity. Increasing evidence suggests that K^+-channel activity is dynamically regulated by the interaction of protein kinases and phosphatases closely associated with the channel complex (Levitan, 1994; Reinhart & Levitan, 1995; White *et al.*, 1993; White *et al.*, 1991). Recent data in AtT20 cells suggest that glucocorticoids recruit a dephosphorylation module with a modified PP2A-like phosphatase activity to the plasma membrane that was closely associated with BK-channels (Tian *et al.*, 1998). Therefore, probing the role of protein phosphorylation in K^+-channel modulation by

glucocorticoids in normal corticotrophs could be invaluable to the understanding of the action of early glucocorticoid inhibition in adenohypophysial corticotrophs.

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Depolarization counteracts glucocorticoid inhibition of adenohypophysical corticotroph cells*

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1 In AtT20 mouse corticotroph tumour cells large conductance Ca^{2+}-activated K^+-channels (BK-channels) have an essential role in the early glucocorticoid inhibition of adrenocorticotrophin (ACTH) secretion evoked by corticotrophin-releasing factor. The present study examined whether or not BK-channels are also pivotal to glucocorticoid inhibition of normal rat anterior pituitary cells.

2 A membrane-permeant, non-metabolizable cyclic AMP analogue, 8-(4-Chlorophenylthio)adenosine-3′,5′-cyclic-monophosphate (CPT-cAMP) was used as the primary secretagogue stimulus, as this mimics the increase of intracellular cyclic AMP caused by corticotrophin-releasing factor, but is not subject to the complex Ca^{2+}-dependent regulation of cyclic AMP metabolism that is evident in corticotroph cells.

3 Experiments in AtT20 cells showed that ACTH secretion stimulated by 1 mM CPT-cAMP was suppressed to $34 \pm 1.5\%$ ($n=12$) of the control stimulus by a maximal dose of 100 nM dexamethasone. The ACTH secretion evoked by the combination of 1 mM CPT-cAMP with either 5 μM (−)BayK8644 (L-type Ca^{2+}-channel activator) or 5 mM TEA (K^+-channel blocker) was respectively $69.1 \pm 7.6\%$ and $69.3 \pm 11.8\%$ of control after 2 h preincubation with 100 nM dexamethasone ($P<0.05$ *vs* CPT-cAMP). The ACTH response elicited by 5 μM (−)BayK8644 and 5 mM TEA given together was completely resistant to inhibition by 100 nM dexamethasone. Furthermore, TEA and (−)BayK8644 given together synergistically stimulated ACTH release in combination with 0.1 mM or 1 mM CPT-cAMP, and these ACTH responses were not inhibited by 100 nM dexamethasone.

4 In primary cultures of rat anterior pituitary cells, TEA (up to 20 mM), charybdotoxin (30 nM) or apamin (100 nM) failed to modify the glucocorticoid inhibition of 0.1 mM CPT-cAMP-induced ACTH release. The combination of 5 mM TEA and 5 μM (−)BayK8644 elicited a small but significant increase in ACTH secretion but did not modify the inhibition of 0.3 mM CPT-cAMP-induced ACTH secretion by 100 nM dexamethasone.

5 In primary cultures of rat anterior pituitary cells, depolarization of the membrane potential with 40 mM KCl enhanced the ACTH response to CPT-cAMP and markedly reduced the maximal inhibitory effect of dexamethasone to $55 \pm 1.2\%$ as well as that of corticosterone to $33 \pm 2.1\%$ *vs* $100 \pm 2.5\%$ and $100 \pm 1.9\%$ inhibition respectively, when 0.1 mM CPT-cAMP was used alone. Introduction of 5 μM (−)BayK8644 with 40 mM KCl in this system had no additional effect on glucocorticoid inhibition.

6 No glucocorticoid inhibition of ACTH release to any of the stimuli applied was observed in cells pretreated with the mRNA synthesis inhibitor, 5,6-dichloro-furanosyl-benzimidazole riboside (DRB) (0.1 mM) or the protein synthesis blocker, puromycin (0.1 mM).

7 In summary, early glucocorticoid inhibition of stimulated ACTH release by cultured rat anterior pituitary cells was dependent on the synthesis of new mRNA and protein. Depolarization of the membrane potential potentiated CPT-cAMP-induced ACTH secretion in AtT20 cells as well as cultured rat corticotrophs and this was associated with a resistance to the early inhibitory effect of glucocorticoids. Glucocorticoid inhibition in rat anterior pituitary corticotrophs was unaltered by TEA, charybdotoxin as well as apamin, and hence it is unlikely to involve predominantly BK-or SK-type Ca^{2+}-activated K^+-channels.

8 These results support the thesis that a prime target of glucocorticoid feedback inhibition in anterior pituitary corticotrophs is the membrane potential and indicate that glucocorticoid-induced proteins regulate the activities of several distinct plasma membrane ion channels.

Keywords: Pituitary gland; potassium channels; dexamethasone; corticosterone; AtT20 cells; stress

Introduction

Adrenal corticosteroids are powerful regulators of gene expression in several organ systems of the body. The plasma levels of these steroids are controlled through a neuroendocrine feedback loop which ensures that corticosteroid levels are optimal for homeostatic adaptation (Dallman *et al.*, 1992). Major sites of corticosteroid feedback action include the anterior pituitary gland, the hypothalamus and further sites in the central nervous system (Sapolsky *et al.*, 1990).

The cellular mechanisms underlying corticosteroid feedback of the hypothalamic-pituitary-adrenocortical system are not well understood. The anterior pituitary corticotroph has served as a useful model to examine this problem (Antoni, 1996; Buckingham, 1996). In corticotroph cells corticosteroid inhibition typically occurs in two main phases, designated early (within 2 h) and late (2–24 h) (Antoni, 1996; Buckingham, 1996). Late inhibition appears to be mediated by negative regulatory elements in DNA and leads to the suppression of

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the proopiomelanocortin (ACTH precursor) (Lundblad & Roberts, 1988), the type 1 corticotrophin releasing-factor (CRF) receptor (Pozzoli *et al.*, 1996) and possibly other genes. In contrast, the early component of inhibition is brought about by glucocorticoid-induced protein(s), the identity of which is unclear at present (Shipston, 1995; Antoni, 1996; Buckingham, 1996).

Recent studies in the mouse corticotroph tumour cell line AtT20 (Shipston *et al.*, 1996) have shown that high conductance Ca^{2+}-activated K^+-channels (BK-channels) play an essential role in the inhibitory effect of dexamethasone on CRF-induced ACTH secretion. Briefly, activation of adenylyl cyclase by CRF produces an increase of cyclic AMP-dependent phosphorylation and triggers Ca^{2+}-dependent action potentials which lead to the exocytotic release of ACTH (see Antoni, 1993). An important element of this stimulatory effect is the inhibition of BK-channels by cyclic AMP-dependent protein kinase (Shipston *et al.*, 1996). Treatment with dexamethasone for 90 min prevented the action of CRF and cyclic AMP analogues on BK-channels through a process requiring the synthesis of mRNA and protein (Shipston *et al.*, 1996). Accordingly, the blockade of BK-channels by charybdotoxin (Antoni, 1996) or iberiotoxin (Shipston *et al.*, 1996) obliterated the inhibitory effect of dexamethasone on stimulated ACTH release.

The aim of the present study was to examine whether BK-channels are also essential for corticosteroid feedback inhibition in non-tumoural corticotroph cells. Experiments were carried out *in vitro*, using AtT20 cells and primary cultures of rat anterior pituitary cells. A cell-permeant cyclic AMP analogue resistant to hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) was used as the stimulus in combination with various compounds that cause membrane depolarization. The rationale for this design was that increases of intracellular cyclic AMP induced by agonists are strongly suppressed by intracellular Ca^{2+} in both AtT20 and normal rat corticotrophs (Antoni *et al.*, 1995; Antoni, 1997), hence changes of cyclic AMP metabolism induced by corticosteroids could mask effects downstream of cyclic AMP formation.

The results clearly show that in contrast to AtT20 cells, blockage of BK-channels fails to counteract early glucocorticoid inhibition in cultures of rat anterior pituitary cells. Importantly, however, corticosteroid inhibition in the rat pituitary culture system was markedly reduced upon depolarization with high extracellular KCl, in which respect its behaviour is analogous to AtT20 cells. Thus, the findings support the notion that early inhibition by glucocorticoids involves control of the membrane potential by rapidly induced proteins.

Methods

Reagents

Unless otherwise indicated all reagents were from Sigma U.K. (Poole, Dorset), and of the highest grade available. The sources of other materials were as follows: CRF (human), ACTH (human) synthetic charybdotoxin (Bachem U.K. Ltd., Saffron Walden); corticosterone (Roussell-UCLAF, Romainville, France); 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic-monophosphate (CPT-cAMP, BIOLOG, Bremen, Germany); tetraethyl ammonium hydrochloride (TEA, Aldrich, Gillingham, Dorset); (−)BayK8644, (RBI, Sigma, U.K.); (+)202-791 (Novartis, U.K.); TRL-3 trypsin (Worthington, Lorne Laboratories, Reading, U.K.); Sheep anti-ACTH serum was generously provided by Prof P.J. Lowry, University of Reading; anti-sheep/goat IgG donkey serum was generously provided by the Scottish Antibody Production Unit, Carluke, Scotland.

Cell culture

Male Wistar rats (120–200 g) were caged singly with free access to pelleted food and tap water in a light- and temperature-controlled environment for 24 h, and sacrificed by decapitation before 10.30 a.m. Precautions were taken to avoid any stressful stimulation of the animals. The anterior pituitary glands were collected and placed in a plastic Petri dish so that the ventral surfaces of the glands contacted the bottom of the dish. The glands were chopped twice (the second time after 90° rotation of the specimen platform) on a hand-operated McIlwain tissue-chopper setting 0.5 mm and lowest blade force. The tissue segments were incubated in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS, GIBCO, Life Technologies, Paisley) supplemented with 25 mM HEPES pH 7.4, 0.15 mM $CaCl_2$, 0.15 mM $MgSO_4$, 0.5 mg ml^{-1} trypsin, 0.01 μg ml^{-1} DNAse I (Sigma D-4527) and 0.25% bovine serum albumin (BSA, Sigma A-7906) for 30 min at 37°C under constant shaking at 250 cycles/min, on a IKA Vibrax orbital shaker. Following trypsinization, 200 μl of 10^4 kallikrein unit/ml Trasylol® (Bayer U.K.) was added and the tissue segments were triturated with a 1 ml Gilson pipette tip attached to the end of a 5 ml polystyrene pipette for 10 min. The suspension was then filtered through 70 μM nylon mesh and centrifuged at 200 *g* for 10 min. The pelleted cells were resuspended in 5 ml HBSS supplemented with 25 mM HEPES pH 7.4, 0.15 mM $CaCl_2$, 0.15 mM $MgSO_4$, 0.01 μg ml^{-1} DNAse I, 0.25 mg ml^{-1} soybean trypsin inhibitor (Sigma T-6522), recentrifuged and suspended in Dulbecco's Modified Eagle's Medium (DMEM, containing 4.5 g glucose/L) supplemented with 2.5% foetal calf serum (Harlan Seralab, Sussex) and 7.5% horse serum (Sigma H-1138). Cell viability as assessed by Trypan Blue exclusion was over 95%. Approximately 5×10^4 cells/well were plated in 24-well plates and cultured in a humidified incubator with 5% CO_2/95% air at 37°C for 4 days.

AtT20 cells were maintained in DMEM (containing 4.5 g/L glucose) supplemented with 10% foetal calf serum as previously described (Woods *et al.*, 1992). All cells used were of passage 30 or less.

Assay of ACTH secretion

The experimental procedures for ACTH secretion were identical for AtT20 cells and primary cultures. The cells were first washed free of serum with 2×0.5 ml of incubation medium (DMEM, GIBCO 31600-026, without Na_2HCO_3, supplemented with 25 mM HEPES pH 7.4 and 0.25% BSA (Sigma, A-4378) and incubated in this medium at 37°C for 1 h, after which steroids and inhibitors of mRNA or protein synthesis were introduced, and incubation was continued for a further 2 h. Steroids were dissolved at 10^{-2} M in dimethylsulphoxide and diluted with incubation medium. Corresponding vehicle controls contained the requisite amounts of dimethylsulphoxide throughout.

In the case of primary cultures, K^+ channel blockers and Ca^{2+}-channel activators were added during the last 30 min of the 2 h preincubation period because normal corticotrophs appear to be relatively resistant to depolarization. After 2 h of preincubation, the wells were washed once more with

incubation medium and the secretagogues and requisite inhibitors were added in 0.5 ml of fresh incubation medium. In some experiments, high KCl medium which was composed of two parts incubation medium and one part 120 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, 25 mM HEPES 0.25% BSA, Ca^{2+}-free medium (120 mM NaCl, 2.4 mM KCl, 1.2 mM K_2HPO_4, 1.2 mM $MgSO_4$, 4.5 g/L D-glucose, 0.2 mM EGTA, 25 mM HEPES, 0.25% BSA, Minimum Essential Medium vitamins and amino acids) or Ca^{2+}-free high KCl medium (two parts of the Ca^{2+}-free medium mixed with one part of 120 mM KCl, 1.2 mM $MgSO_4$, 0.2 mM EGTA, 25 mM HEPES, 0.25% BSA, vitamins and amino acids) were used. The test incubation period was 60 min for primary cultures and 30 min for AtT20 cells after which the tissue culture trays were placed on ice. Any cells detached from the bottom of the wells were pelleted by centrifugation at 200 *g* for 10 min at 4°C after which aliquots of the supernatant were collected and assayed for ACTH content by radioimmunoassay. Sheep anti ACTH serum used in the assay was generously provided by Prof P.J. Lowry, University of Reading and the donkey antisheep IgG serum was obtained from the Scottish Antibody Production Unit, Carluke, Scotland.

Analysis of results

Each experiment was carried out at least twice with **n** replicates for each treatment within each experiment. All data are presented as mean ± s.e.mean and were analysed by 2- or 1-way analysis of variance followed by Dunnett's test, Newman Keuls' test or contrast of means where appropriate. Results presented as percentage of control ACTH release were derived from the raw data as follows: $(X-B)/(C-B) \times 100\%$, where B refers to the basal ACTH secretion, C refers to ACTH secretion elicited by the control stimulus and X refers to ACTH secretion elicited by the control stimulus in the presence of dexamethasone or corticosterone.

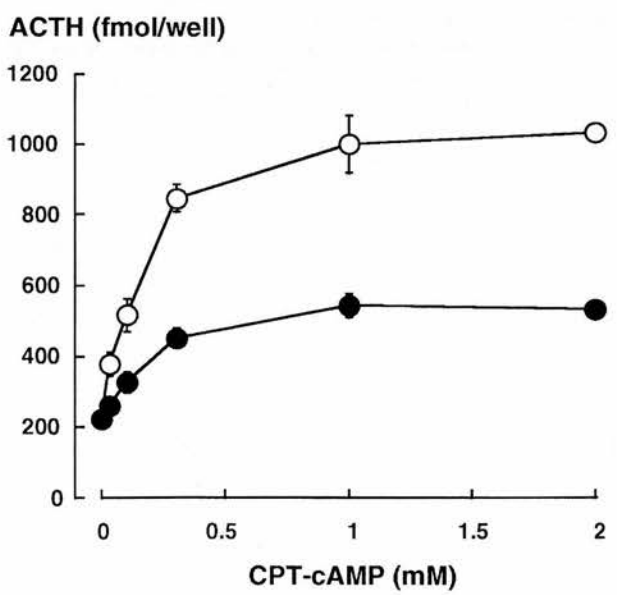

Figure 1 The effect of CPT-cAMP on ACTH secretion by AtT20 cells pretreated for 2 h with vehicle (○) or 100 nM dexamethasone (●). Means, the bars indicate s.e.mean, $n=5$/group. Where error bars are not visible, the s.e.mean bar is less than the space required for the symbol. Results shown are representative of four identical experiments.

Results

Experiments with AtT20 cells

Response to CPT-cAMP and dexamethasone Concentration-dependent stimulation of ACTH release was evoked by CPT-cAMP (Figure 1) and the response plateaued at 1 mM CPT-cAMP. Pretreatment with 100 nM dexamethasone significantly reduced the increase in CPT-cAMP-stimulated ACTH secretion, basal hormone output remained unaltered (Figure 1).

Effects of depolarizing agents The ACTH response to 1 mM CPT-cAMP could be enhanced further by the application of

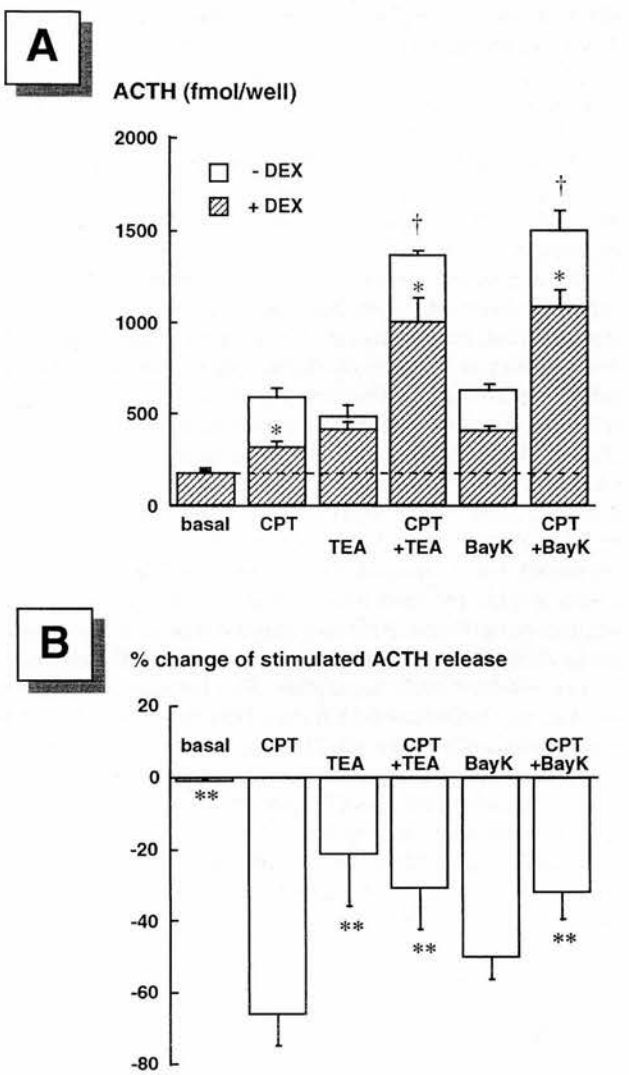

Figure 2 Effect of membrane depolarizing agents, 5 μM (−)BayK8644 (BayK) and 5 mM tetraethylammonium (TEA) on ACTH secretion elicited by 1 mM CPT-cAMP (CPT) after 2 h preincubation with vehicle or 100 nM dexamethasone in AtT20 cells. Data are means, bars indicate s.e.mean, $n=4$/group. (A) The empty columns show ACTH release by vehicle treated cells and the hatched columns show release after treatment with dexamethasone. †$P<0.02$ for the interaction between CPT alone and the respective depolarizing agents alone, 2-way ANOVA. *$P<0.05$ when compared to corresponding vehicle-treated release, 1-way ANOVA followed by contrast of means. (B) data are expressed as % change in the amount of ACTH secretion caused by dexamethasone when compared with the respective control groups shown in A. **$P<0.05$ when compared with CPT alone. 1-way-ANOVA followed by Dunnett's test. Results shown are representative of two identical experiments.

5 mM tetraethylammonium (TEA) or the Ca^{2+}-channel activator (−)BayK8644 at 5 μM (Figure 2A). Both of these compounds stimulated ACTH release when given alone and exhibited synergistic interaction with CPT-cAMP. Similar data were obtained with another L-channel activator (+)202−791 at 5 μM (not shown).

The release of ACTH evoked by 1 mM CPT-cAMP was inhibited by 65.8±8.9% ($n=5$) by 100 nM dexamethasone, while basal secretion was not significantly altered (Figure 2A and B). The inhibition of ACTH secretion stimulated by a combination of 1 mM CPT-cAMP and 5 mM TEA or 1 mM CPT-cAMP and 5 μM (−)BayK8644 was significantly attenuated to 30.7±11.8% and 31.9±7.6% ($P<0.05$, $n=5$), respectively (Figure 2A and B). The combination of 1 mM CPT-cAMP with 5 μM (+)202−791 also significantly reduced the inhibition of the stimulated ACTH secretion by 100 nM dexamethasone to 27±1.3% ($P<0.05$, $n=4$/group, 1-way-ANOVA, Dunnett's test).

As both (−)BayK8644 and TEA partially reversed dexamethasone inhibition of CPT-cAMP stimulated ACTH secretion, the combination of these compounds with CPT-cAMP was tested subsequently, using a submaximal concentration (0.1 mM) of CPT-cAMP as well as 1 mM CPT-cAMP. The ACTH secretion elicited by 0.1 mM CPT-cAMP was significantly potentiated by 5 μM (−)BayK8644 and 5 mM TEA given together (Table 1). CPT-cAMP-induced ACTH secretion was inhibited by dexamethasone (1 nM to 1 μM) in a concentration dependent manner by up to 84% (Table 1). The combination of 5 μM (−)BayK8644 and 5 mM TEA elicited a 3-fold increase in ACTH secretion over basal levels and this response was not inhibited by dexamethasone (Table 1). The ACTH response elicited by the combination of 0.1 mM CPT-cAMP, 5 μM (−)BayK8644 and 5 mM TEA was also fully resistant to inhibition by 1−1000 nM dexamethasone (Table 1).

Similar results were obtained by combining 1 mM CPT-cAMP with 5 mM TEA and 5 μM (−)BayK8644. A summary of these data (Figure 3) shows that the extent of resistance to glucocorticoid inhibition was independent of the amplitude of stimulus-evoked ACTH secretion. For instance, although the ACTH response elicited by 5 mM TEA or 1 mM CPT-cAMP alone were similar, the ACTH response to 5 mM TEA was much more resistant to dexamethasone inhibition than the ACTH response to 1 mM CPT-cAMP.

Taken together, these results are in agreement with previous studies of CRF-induced ACTH release in AtT20 cells, which have shown that early glucocorticoid inhibition is effectively antagonized by depolarization of the membrane potential (Woods *et al.*, 1994) and that TEA sensitive K^+-channels, particularly BK-type channels underlie the inhibitory action of corticosteroids (Shipston *et al.*, 1996).

Experiments with primary cultures of rat anterior pituitary cells

Response to CPT-cAMP The time-course of the ACTH response to CPT-cAMP was close to linear over 4 h (Figure 4A). The concentration-response relationship between CPT-cAMP and ACTH release was similar at 60, 120 and 240 min, in that 0.1 mM CPT-cAMP elicited close to maximal

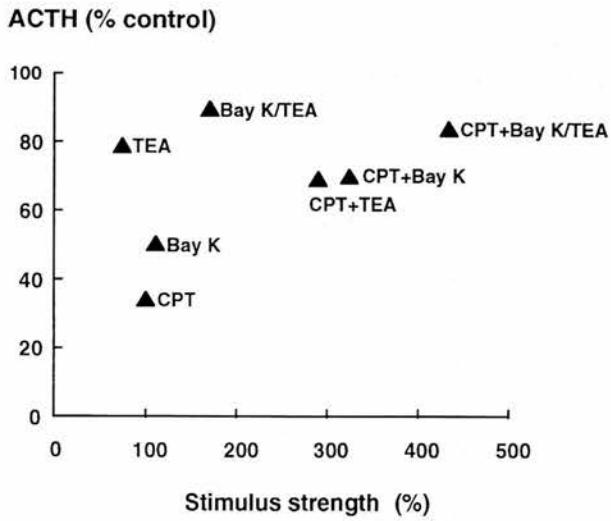

Figure 3 Size of the ACTH secretory response fails to predict the degree of dexamethasone inhibition in AtT20 cells. Data were plotted as stimulus strength *vs* ACTH response, where stimulus strength is the size of the evoked release of ACTH expressed as % of the response to 1 mM CPT-cAMP run in the same experiment, and ACTH response is the size of the ACTH response to the respective stimulus in cells preincubated with 100 nM dexamethasone, expressed as percentage of the control (i.e. no dexamethasone) response. Data are means $n=4$/group. Symbols: CPT, 1 mM CPT-cAMP; BayK, 5 μM (−)BayK8644; TEA, 5 mM tetraethylammonium; BayK/TEA, combination of 5 μM (−)BayK8644 and 5 mM tetraethylammonium; + sign indicates that two stimuli were applied together. Results shown are representative of two identical experiments.

Table 1 Characterization of the ACTH secretory response to CPT-cAMP, TEA and (−)BayK8644 in the presence of increasing dexamethasone concentrations in AtT20 cells

	Dexamethasone (nM)				
	0	*3*	*10*	*100*	*1000*
Vehicle	608±63				
0.1 mM CPT	1122±136	860±94	795±57	734±19	690±22
	(100±26%)	(49±18%)	(36±11%)	(25±4%)	(16±4%)
5 mM TEA +	1883±65	1973±125	1767±57	1783±84	1743±167
5 μM BayK	(100±5%)	(107±10%)†	(91±4%)†	(92±7%)†	(89±13%)†
0.1 mM CPT +	2919±93*	2912±126	2593±170	2626±62	3016±161
5 mM TEA +	(100±4%)	(100±5%)†	(86±7%)†	(87±3%)†	(104±7%)†
5 μM BayK					

Data show ACTH release expressed in fmol per well and represent means±s.e.mean. of four replicates per treatment group. Values in parentheses represent percentages of control ACTH release (mean±s.e.mean), as defined in Methods. *$P<0.02$ for the interaction between CPT and TEA/BayK in the absence of dexamethasone pretreatment, 2-way ANOVA. Statistical analysis of the effect of dexamethasone was carried out on the data expressed as percentages (values shown in parentheses) of the respective stimulus-evoked release, †$P<0.05$ when compared to the corresponding CPT group, 1-way ANOVA followed by Dunnett's test. Results shown are representative of two identical experiments.

stimulation of ACTH release (Figure 4B). This was not the case at 30 min, where 1 mM CPT-cAMP was significantly more effective than 0.1 mM CPT-cAMP. All subsequent experiments were terminated at the 60 min time-point.

Lack of an effect of K^+-channel blockers and BayK8644 on corticosteroid inhibition of ACTH release Corticosterone (100 nM) reduced the ACTH response to 0.1 mM CPT-cAMP by 74.8±10.8% ($n=5$) and this was not modified in the presence of TEA (5–20 mM) or (−)BayK8644 (5 μM). Similar findings were obtained with 30 nM dexamethasone. Combination of 5 mM TEA and (−)BayK8644 (5 μM) produced a significant enhancement of the ACTH releasing effect of CPT-cAMP (0.3 mM) (Basal: 34±6 fmol/well; CPT-cAMP: 125±5 fmol/well, $P<0.0001$, $n=6$; TEA+BayK: 60±6 fmol/well, $P<0.0002$, $n=5$; CPT-cAMP+TEA+BayK: 204±14 fmol/well, $P<0.03$, interaction, $n=7$, 2-way-ANOVA) but failed to modify the inhibition by 100 nM dexamethasone (% inhibition of CPT-cAMP *vs* CPT-cAMP+TEA+BayK: 63±7% *vs* 66±6%, $n=4$). Finally, preincubation with charybdotoxin (30 nM) or apamin (100 nM) and the combination of these agents with 0.1 mM CPT-cAMP also failed to modify corticosterone inhibition of stimulated ACTH secretion (not shown).

These data indicate that in contrast to AtT20 cells, TEA-sensitive ion-channels are not fundamentally important for corticosteroid inhibition of ACTH secretion in primary cultures of rat anterior pituitary cells.

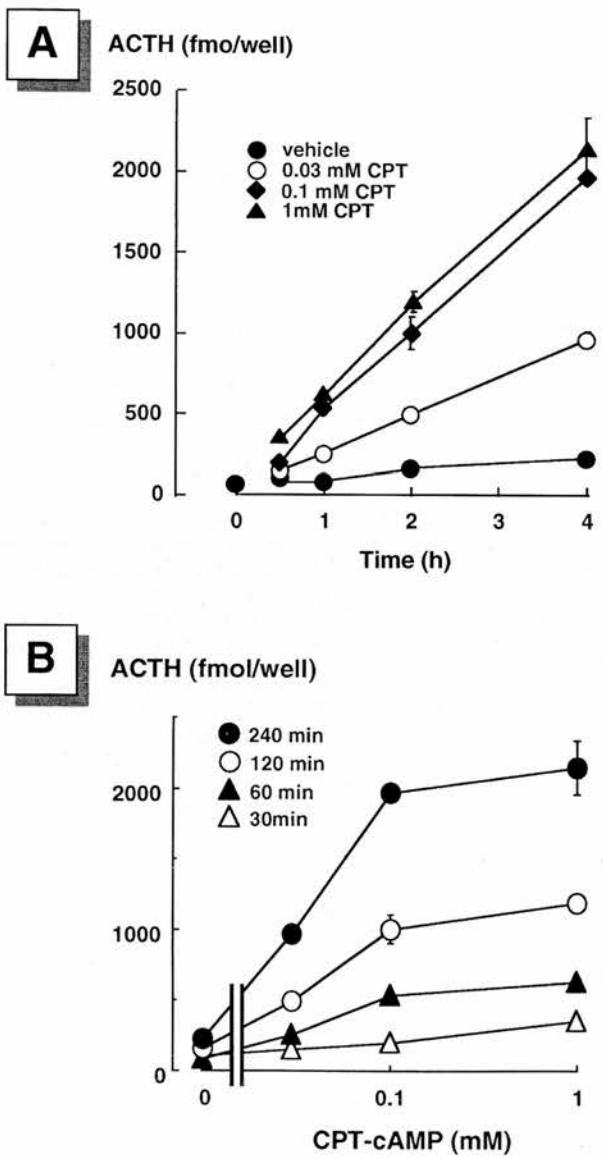

Figure 4 Effect of CPT-cAMP on ACTH secretion by primary cultures of rat anterior pituitary cells. (A) Time-course of hormone release in cells treated with vehicle (●), 30 μM CPT-cAMP (○), 0.1 mM CPT-cAMP (◆) or 1 mM CPT-cAMP (▲). (B) Concentration-dependent stimulation of ACTH secretion by CPT-cAMP at 30 min (△), 1 h (▲), 2 h (○) and 4 h (●) of incubation. Means, bars indicate s.e.mean, $n=4$/group. Where error bars are not visible, the s.e.mean bar is less than the space required for the symbol. Results shown are representative of two identical experiments.

Depolarization with 40 mM KCl counteracts glucocorticoid effect on ACTH The ACTH responses to 0.1 mM CPT-cAMP and 40 mM KCl are shown in (Table 2). KCl (40 mM) synergized with 0.1 mM CPT-cAMP to stimulate ACTH release, furthermore, it produced a marked reduction in the inhibitory effect of dexamethasone (Table 2). Omission of Ca^{2+} from the incubation medium completely blocked ACTH release induced by CPT-cAMP alone or CPT-cAMP and KCl (not shown). Dexamethasone (10 nM) inhibited the ACTH response to 0.1 mM CPT-cAMP and 40 mM KCl by only 44±12% ($n=4$), while that evoked by 0.1 mM CPT-cAMP was completely blocked. Similar results were also obtained using the naturally occurring corticosteroid corticosterone (Table 2).

The data above demonstrate that depolarization of the membrane potential markedly reduces the early inhibitory effect of glucocorticoids. However, the lack of a complete block of steroid action by 40 mM KCl raises the question of whether or not the inhibitory effect may be completely accounted for by the rapid induction of mRNA and protein by glucocorticoids.

Blockers of mRNA and protein synthesis abolish glucocorticoid inhibition The inhibition of 0.1 mM CPT-cAMP stimulated ACTH secretion by 10 nM dexamethasone was eliminated in the presence of 5,6-dichloro-furanosyl-benzimidazole riboside (DRB), an adenosine analogue and inhibitor of heteronuclear RNA synthesis (Egyházi *et al.*, 1982) (Figure 5A). In the presence 40 mM KCl, DRB suppressed ACTH secretion elicited by 0.1 mM CPT-cAMP but no further change was observed after treatment with dexamethasone (Figure 5B). Therefore, the residual inhibition by corticosteroids observed in the presence of 40 mM KCl and 0.1 mM CPT-cAMP is likely to be due to glucocorticoid blockade of this DRB sensitive mechanism. Similar results were obtained using 0.1 mM puromycin, an inhibitor of protein synthesis (not shown).

Discussion

The data presented here show that control of the membrane potential is essential for glucocorticoid inhibition of stimulated ACTH release in both AtT20 cells and primary cultures of rat anterior pituitary cells. However, in contrast to tumoural AtT20 cells where BK-channels are pivotal targets of glucocorticoid action (Shipston *et al.*, 1996), blockage of BK- and other TEA-sensitive K^+-channels failed to modify the inhibitory action of glucocorticoids in normal rat pituitary cells. Thus the data conform with the general hypothesis that glucocorticoids modulate the function of plasma membrane ion channels to reduce cellular responses of excitable cells (Joëls & de Kloet, 1994), and indicate that glucocorticoid-

Table 2 Counteraction of glucocorticoid inhibition of the ACTH secretory response by 40 mM KCl in primary cultures of rat anterior pituitary cells

	Corticosterone (nM)				
	0	*3*	*10*	*100*	*1000*
Vehicle	100.4±11.0				
0.1 mM CPT	163.8±6.9 (100±11%)	150.8±8.1 (80±13%)	132.6±15.2 (51±24%)	86.4±2.1 (−22±3%)	80.3±3.0 (−31±5%)
40 mM KCl	125.5±2.9				
0.1 mM CPT + 40 mM KCl	284.8±4.1* (100±2%)	269.8±16.3 (92±9%)	260.8±7.6 (87±4%)†	221.9±23.1 (66±13%)†	216.3±22.7 (63±12%)
	Dexamethasne (nM)				
	0	*3*	*10*	*100*	
Vehicle	66.1±7.0				
0.1 mM CPT	112.8±5.3 (100±11%)	62.8±2.5 (−7±5%)	54.5±3.3 (−25±7%)	52.0±3.1 (−30±7%)	
40 mM KCl	127.6±14.4				
0.1 mM CPT + 40 mM KCl	280.6±27.2** (100±13%)	192±31.8 (59±15%)†	187.2±26.1 (56±12%)†	165.2±11.8 (46±6%)†	

Data show ACTH release expressed in fmol per well and represent means±s.e.mean. of four replicates per treatment group. Values in parentheses represent percentages of control ACTH release (mean±s.e.mean), as defined in Methods. *$P<0.0001$ and **$P<0.004$ for the interaction between CPT-cAMP and KCl in the absence of glucocorticoid pretreatment, 2-way ANOVA. Statistical analysis of the effect of glucocorticoids was carried out on the data expressed as percentages (values shown in the parentheses) of the respective stimulus-evoked release, †$P<0.05$ when compared to the corresponding CPT group, 1-way ANOVA followed by contrast of means. Results shown are representative of two and three identical experiments for the corticosterone and dexamethasone dose responses respectively.

induced proteins may regulate the function of multiple plasma membrane ion channels.

Characteristics of the primary culture system as a model for secretagogue-glucocorticoid interaction and the use of CPT-cAMP

Previous studies have established that the main hallmarks of early glucocorticoid inhibition are: (i) selectivity for stimulated ACTH release, (ii) mediation through Type II glucocorticoid receptors, (iii) manifestation within 2 h, and (iv) a requirement for new mRNA and protein synthesis reviewed in (Shipston, 1995). These features have been largely retained in the AtT20 D16:16 cell line (Antoni, 1996), and also characterize the rat anterior pituitary primary culture system (Abou-Samra *et al.*, 1986, and present study).

A further important property of the primary culture system is its responsiveness to physiological concentrations of CRF and AVP (Vale *et al.*, 1983; Oki *et al.*, (1990)). A somewhat surprising feature is the low response to depolarizing stimuli such as 40 mM KCl and BayK8644 given alone. These stimuli have been reported as inducers of robust ACTH release by acutely prepared pituitary cells (Gillies & Lowry, 1978; Antoni & Dayanithi, 1990b), tissue segments (Taylor *et al.*, 1993) as well as AtT20 cells (Guild & Reisine, 1987). A plausible explanation of this finding is that the aforementioned preparations possess spontaneous electrical activity, which may be due to endogenous pacemaker potentials (e.g. AtT20 cells), or is derived from factors released upon tissue injury due to dissection in pituitary segments (Taylor *et al.*, 1993), or is a remnant of the trophic actions of CRF *in vivo* (Ixart *et al.*, 1991). Spontaneous electrical activity will facilitate the effects of the usage-dependent L-channel activator (−)BayK8644 (Sanguinetti & Kass, 1984). Furthermore, the secretagogue potencies of (−)BayK8644 as well as high KCl are likely to depend on tonic cyclic AMP-dependent phosphorylation which is required for the optimal functioning of L-type Ca^{2+} channels (Armstrong & Eckert, 1987) and exocytotic processes (Morgan *et al.*, 1993).

With respect to the requirement for cyclic AMP, it is known that acutely dispersed cells produce much more reliable responses to short pulses CRF if 'primed' with several pulses of the peptide (Antoni *et al.*, 1990b). Moreover, it has been reported that cyclic AMP and Ca^{2+} synergize to trigger ACTH release from permeabilized AtT20 cells (Guild, 1991), further indicating that cyclic AMP-dependent processes acting in parallel to the mobilization of Ca^{2+} are important for the secretory response of corticotroph cells. It is of note that while depolarization with 40 mM KCl was not a reliable stimulus of ACTH release on its own, it consistently produced a synergistic enhancement of the CPT-cAMP induced ACTH response. As the main result of KCl depolarization is Ca^{2+} influx through voltage-operated channels (Meier *et al.*, 1988), the synergy of CPT-cAMP and KCl depolarization appears analogous to the Ca^{2+} potentiation of cAMP-induced ACTH release in AtT20 cells. Similar results have been obtained using physiological concentrations of CRF (Lim *et al.*, unpublished).

A cell- membrane permeant, non-metabolizable analogue of cyclic AMP, CPT-cAMP, was used as the secretagogue stimulus in this study in order to produce a sustained increase in cyclic AMP-dependent phosphorylation. As the physiological ACTH secretagogues, CRF or CRF and AVP in combination, activate cyclic AMP-dependent phosphorylation in corticotrophs (see Antoni, 1993), CPT-cAMP will mimic this aspect of agonist action. It is also clear that CRF-induced cyclic AMP responses are subject to negative feedback regulation by intracellular free Ca^{2+} at the level of adenylyl cyclase and cyclic AMP hydrolysis in AtT20 cells (Antoni, 1996) as well as rat anterior pituitary cells (Antoni, 1997). CPT-cAMP is largely resistant to Ca^{2+}-feedback and this property makes it a potentially useful tool for studying processes of ACTH release downstream of cyclic AMP formation. It is of note, however, that CRF elicits oscillations of intracellular free Ca^{2+} in AtT20 cells as well as normal rat corticotrophs, (see Antoni, 1993 for review), which may be accompanied by oscillations of intracellular cyclic AMP levels (Cooper *et al.*, 1998). Thus, clamping the intracellular cyclic AMP through CPT-cAMP, which leads to sustained increases of intracellular free Ca^{2+} as opposed to oscillations (Mollard

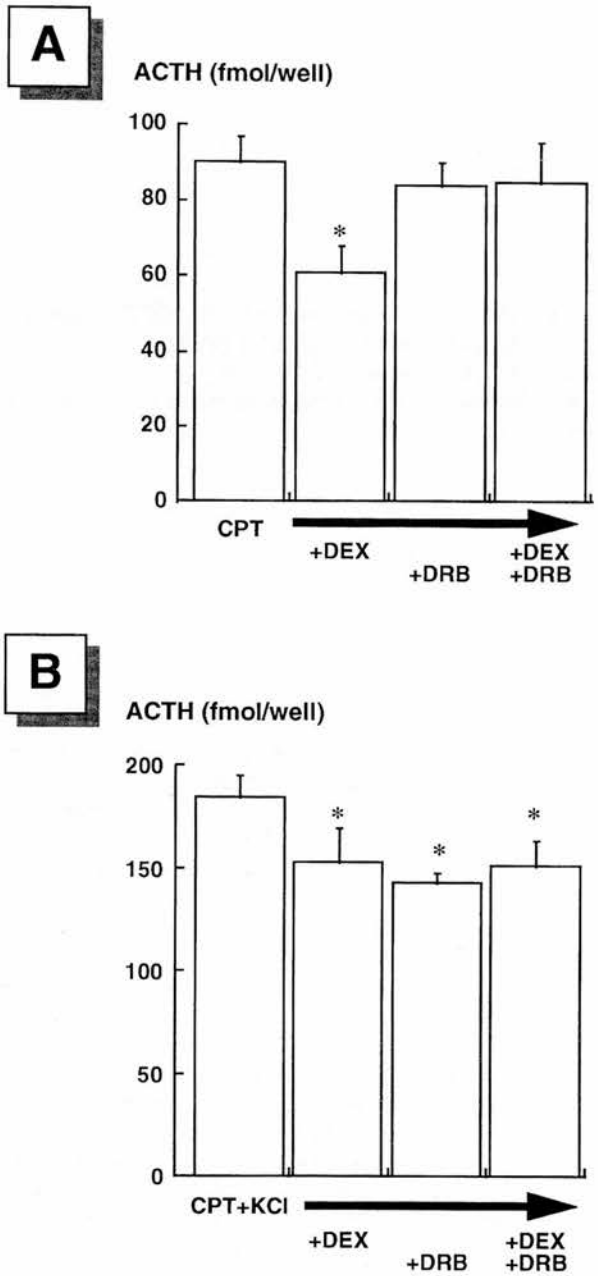

Figure 5 Effect of the RNA synthesis inhibitor 5,6-dichloro-furanosyl-benzimidazole riboside (DRB) on ACTH secretion in primary cultures of rat anterior pituitary cells. 100 μM DRB and 100 nM dexamethasone (DEX) were applied 2 h before challenging the cells with (A) 0.1 mM CPT-cAMP or (B) 0.1 mM CPT-cAMP and 40 mM KCl for 60 min. Basal ACTH release was 44 ± 3 fmol/well and was not altered by DRB or dexamethasone. Data are means, bars indicate s.e.mean, $n = 5$/group. $^*P < 0.05$ when compared with control group receiving vehicle only. 1-way-ANOVA followed by Newman-Keuls test. Results shown are representative of four identical experiments.

et al., 1992), may potentially alter some properties of the ACTH response when compared with physiological agonists. Indeed, inhibition of mRNA or protein synthesis significantly attenuated the release of ACTH by rat pituitary corticotrophs to CPT-cAMP-based stimuli, whereas such manipulations were without effect on CRF-induced ACTH secretion (Dayanithi & Antoni, 1989; Taylor *et al.*, 1993 and Lim *et al.*, unpublished). In other respects, however, CPT-cAMP-stimulated ACTH release was largely dependent on extracellular Ca^{2+} and blocked by glucocorticoids similarly to ACTH secretion evoked by CRF.

Properties of early glucocorticoid inhibition in AtT20 cells

The results with AtT20 cells extend previous work from this laboratory examining CRF-induced ACTH release in these cells. Overall, ACTH release induced by CPT-cAMP alone was potently inhibited by dexamethasone. By contrast, responses to stimuli that cause depolarization such as TEA, (−)BayK8644 or (+)202−791 were resistant to glucocorticoid inhibition. It was also clear that the nature of the stimulus rather than the size of the evoked response predicted the degree of glucocorticoid inhibition in AtT20 cells. Thus, these data are in full agreement with earlier findings from this laboratory indicating that the principal target of corticosteroid inhibition is the membrane potential (Pennington *et al.*, 1994; Shipston *et al.*, 1996) rather than the secretory apparatus as proposed by others (Castellino *et al.*, 1992). Further, the data are in agreement with the findings that BK-channels (Shipston *et al.*, 1996) are pivotal for glucocorticoid inhibition in AtT20 cells.

Corticosterone action in primary cultures: deviation from and convergence to AtT20 model

Importantly, the involvement of plasma membrane ion-channels in glucocorticoid action has not been previously addressed in normal rat corticotrophs. A report from another laboratory has shown (Halili-Manabat *et al.*, 1995) that ACTH secretion stimulated by the depolarizing agent veratridine, an opener of tetrodotoxin sensitive Na^+-channels is resistant to inhibition by dexamethasone, and our group has reported similar findings in preliminary form using combinations of CRF and veratridine (Antoni & Woods, 1992). However, no stimuli physiological to corticotrophs have been identified so far that operate through Na^+-channels and thus counteract glucocorticoid feedback inhibition (Antoni, 1993; Halili-Manabat *et al.*, 1995).

In sharp contrast to AtT20 cells, concentrations of TEA as high as 20 mM failed to dent significantly the early inhibitory action of corticosterone or dexamethasone in primary cultures of rat anterior pituitary cells. Also of note is that apamin and charybdotoxin, blockers of SK- and BK-type Ca^{2+}-activated K^+-channels were also without effect. In contrast to the channel blockers tested so far, 40 mM KCl synergized with CPT-cAMP to stimulate the release of ACTH, while having relatively little effect on its own. Furthermore, the early inhibitory effects of dexamethasone as well as corticosterone were drastically reduced with CPT-cAMP and KCl as the stimulus. The release of ACTH under these conditions was fully dependent on extracellular Ca^{2+}, hence indicating that the mechanism of hormone release is not fundamentally altered by the addition of 40 mM KCl.

Overall, these findings are surprising, as channels sensitive to the K^+-channel blockers applied in the present study, and functionally relevant for the regulation of ACTH release have been previously reported in various preparations of corticotroph cells (Marchetti *et al.*, 1987; Antoni & Dayanithi, 1990a). The possibility that the K^+-channel profile of corticotrophs is altered by culture conditions cannot be discounted at present. A recent report (Lee & Tse, 1997) suggests that CRF-induced activation of cultured rat pituitary corticotrophs is the result of the inhibition of a K^+-current that is insensitive to TEA as well as 4-aminopyridine. However, as noted above, the role of K^+

Table 3 Comparison of early corticosteroid inhibition of stimulated ACTH release in AtT20 cells and primary cultures of rat anterior pituitary cells

Common features:
Onset within 2 h
Mediated by Type II glucocorticoid receptor
Requirement of new mRNA and protein synthesis
Specific for agonist-induced release
Opposed by depolarization of the membrane potential
Specific features:
AtT20 cells
Mediated by BK-type potassium channels
Primary cultures
Mediated by unidentified ion channel
May involve transcriptional block of protein expression required for sustained release of ACTH

channels in glucocorticoid feedback has not been previously examined in non-tumoural corticotrophs.

The nature of the residual inhibitory effect of glucocorticoids on KCl/CPT-cAMP stimulated ACTH release was revealed in experiments with inhibitors of protein and mRNA synthesis. As some reports suggested that early corticosteroid inhibition does not require gene induction (Taylor *et al.*, 1993) we have re-examined this issue in the primary culture system. Similarly to actinomycin D (Arimura *et al.*, 1969; Portanova & Sayers, 1974; Dayanithi *et al.*, 1989) DRB blocked the early corticosteroid inhibition of ACTH release, indicating a requirement for mRNA synthesis. Moreover, DRB as well as puromycin caused similar reductions in ACTH release induced by 40 mM KCl/0.1 mM CPT-cAMP in the presence of 100 nM dexamethasone, which had no additional effect in the presence of these agents. The findings indicate that in case of relatively high (>4 fold basal) ACTH responses elicited by CPT-cAMP-based stimuli the ACTH secreted is derived through the synthesis of new mRNA and protein, plausibly POMC, which is blocked by glucocorticoids. In contrast to CPT-cAMP-based stimuli, there was no apparent requirement for mRNA or protein synthesis when CRF was used to stimulate ACTH release (Abou-Samra *et al.*, 1986; Dayanithi *et al.*, 1989; Taylor *et al.*, 1993). Thus oscillating Ca^{2+}/cAMP signals may be more efficient in mobilizing/processing ACTH without a requirement for *de novo* protein synthesis than CPT-cAMP which elicits prolonged elevations of intracellular Ca^{2+} lacking an oscillatory pattern (Mollard *et al.*, 1992).

In summary (Table 3), it is intriguing that the same physiologically relevant phenotype of early corticosteroid inhibition is found in AtT20 cells and rat corticotrophs, including counteraction by membrane depolarization, but the underlying membrane-control mechanisms have strikingly distinct pharmacology. Further studies should clarify whether glucocorticoids are capable of inducing different families of ion-channel subunits depending on the cell system under study (Attardi *et al.*, 1993; Levitan *et al.*, 1996) or evoke the synthesis of proteins that regulate the activity of several distinct ion channels through generic mechanisms such as protein phosphorylation (Wang *et al.*, 1997). Data from our laboratories (Shipston *et al.*, 1996) favour the latter possibility in the case of adenohypophysial corticotrophs.

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